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Journal of Virological Methods 165 (2010) 283–293

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Multiplex nested RT-PCR for the detection of porcine enteric viruses

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Article history:
Received 16 July 2009
Received in revised form 5 February 2010
Accepted 9 February 2010
Available online 17 February 2010

Keywords:
Coronavirus
Rotavirus
RT-PCR
Immunochromatographic test strip

A B S T R A C T

Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine group A rotavirus (PRV-A) are major viruses causing enteric diseases of piglets. A multiplex nested reverse transcription polymerase chain reaction (multiplex nested RT-PCR) was developed for the detection of these viruses in field samples from piglets with diarrhea. A mixture of (1) three external pairs of primers, yielding in the amplification step two different amplicons with sizes of 950 bp and 317 bp and (2) three pairs of internal primers in a second round of PCR (nested PCR), yielding two different amplicons with sizes of 792 bp and 208 bp for TGEV and porcine PRV-A, respectively. The genome of PEDV was not detected after the amplification step but it was detected in the second round of PCR, yielding amplicon with size of 291 bp. Multiplex nested RT-PCR can detect TGEV, PRV-A, and PEDV up to concentration 10^2 $TCID_{50}$/mL, 10^1 $TCID_{50}$/mL, and 27.2 μg/μl of RNA, respectively. A total of 175 field samples were collected from swine with diarrhea from January 2005 until July 2007. The samples were tested for the presence of three viruses by a multiplex nested RT-PCR. Dual infections with PEDV and PRV-A were identified in seven specimens (4%) ($n = 6$). Twenty-one (25%) infections were caused by PEDV and thirty-four infections (41%) were caused by PRV-A. The genome of TGEV was not detected in any of these field samples, however TGEV was detected in piglets infected experimentally. The multiplex nested RT-PCR is rapid, sensitive, and a cost-effective detection method for the detection of porcine enteric viruses.

1. Introduction

Acute infectious diarrhea is a major cause of high morbidity and mortality in piglets. Group A rotaviruses (PRV-A), transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) are recognized as the principal viruses causing gastroenteritis in piglets. PEDV and TGEV are members of the *Coronaviridae* family and cause watery diarrhea, dehydration, and high mortality in suckling pigs. Rotaviruses, members of the *Reoviridae* family, are the most common cause of porcine gastroenteritis. The TGEV, PEDV, and PRV-A infections are difficult to differentiate clinically (Cavanagh, 1997; Pensaert, 1999; Saif and Wesley, 1999); these viruses cause enteritis in swine of all ages, and are fatal frequently in neonatal piglets, resulting in serious economic loss. They induce similar clinical signs and lesions: destroy villous enterocytes and cause villous atrophy in the small intestine, resulting in profuse diarrhea and vomiting. Hence, it is important to develop an effective and rapid method for their differential detection (Rodák et al., 2004). The development of duplex RT-PCR, either classical or quantitative, for the detection of porcine enteric viruses has been described by many investigators (Kim et al., 2000, 2001, 2007; Jung and Chae, 2005). Although these methods may contribute to differential detection of two given viruses, some of these methods depend on the laboratory facilities. Furthermore, real time PCR is a cost-effective assay for routine research. Song et al. (2006) described the development of a multiplex RT-PCR for rapid differential detection of PEDV, TGEV, and PRV-A using pairs of specific primers published previously (Kim et al., 2001; Elschner et al., 2002) which may be useful for routine diagnosis.

A multiplex nested RT-PCR method using specific primers designed for the detection and differentiation of TGEV, PEDV, and PRV-A in field samples taken from Russian swine farms was developed, and the nucleotide analysis of Russian PEDV isolates by molecular sequencing of the amplified product is described.

2. Materials and methods

2.1. Viruses

Three strains were used in this study: cell culture adapted "Leningradsky" strain (10^8 $TCID_{50}$/mL) of TGEV and cell culture adapted "Krasnadonsky" strain (10^8 $TCID_{50}$/mL) of TGEV, cell cul-

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doi:10.1016/j.jviromet.2010.02.010

ture adapted "KR-47" strain (10^6 $TCID_{50}$/mL) of PRV-A, available in the Russian State Collection of microorganism strains (Federal Governmental Institute, All-Russian State Center for Quality and Standardization of animal medicines and feeds of Russian Ministry of Agriculture, FGI VGNKI) under registration numbers "Leningradsky-ARRIAH-DEP", "Krasnadonsky-ARRIAH-DEP", "KR-47-ARRIAH-DEP", respectively. PEDV positive stool samples were also used in this study.

2.2. RNA extraction

Viral RNA extracted from PEDV, TGEV, PRV-A, cultured in cells and from pathological and stool specimens were carried out according to the procedure of Gribanov et al. (1997) using GF/F filters as described previously with modifications. Briefly, 100 μL of the sample suspension was mixed with 200 μL of 4 M guanidine thiocyanate (GTC) (Promega, Washington, USA). The mixture was incubated during 10 min at 56 °C. 300 μL of 96% ethanol was added, mixed, and the mixture was passed through glass fiber filter GF/F (Whatman, England, UK) under vacuum (Vac-Man® Laboratory Vacuum Manifold, Promega, Washington, USA). The filters were washed three times with 800 μL of 80% ethanol. The remaining ethanol was removed by centrifugation at 5000 × *g* for 10 s. The RNA was eluated from the filters with 50 μL of deionized water. The RNA was then stored at −80 °C or used immediately.

2.3. Propagation of PEDV in cell culture

The PEDV isolates were propagated in Vero cell monolayers grown in alpha-MEM supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth, and 2 mg/mL of trypsin as described previously (Kweon et al., 1994, 1999; Hofmann and Wyler, 1988; Kusanagi et al., 1992). Vero cell cultures were examined daily for cytopathic effects (CPE). If the cell layer did not show CPE after 5 days of incubation, cells and supernatant fluids were frozen and thawed three times to release intracellular virus into the medium. The fluid was clarified by low-speed centrifugation (300 × *g* for 15 min).

2.4. Sample collection

A total of 175 samples were collected from 83 piglets with gastroenteritis from seventeen different swine farms in nine different Russian regions between January 2005 and July 2007. All samples were classified according to the stage of infection of the animals. During acute infection, 28 fecal samples were collected; 37 fecal, intestine, and lung–spleen samples were collected during the acute infection and post-mortem; 18 intestine and lung–spleen samples were collected from each animal after death or post-mortem. These samples included in total 65 feces, 55 intestine, and 55 lung/spleen samples.

Approximately 10% stool or 30% homogenized tissue suspensions were mixed with bidistilled water and clarified by centrifugation at 300 × g for 10 min at 4 °C. The supernatant was stored at −80 °C until use.

All data were processed and analyzed by Epi Info (TM) 3.5.1 (Dean et al., 2007). Differences between groups are considered significantly different for $P < 0.05$.

2.5. Chromatographic immunoassay

The detection of PRV-A, PEDV, and TGEV antigens in clinical samples was carried out using a commercial kit (ANIGEN Rapid PED Ag Test Kit, Catalogue No. RG14-01; ANIGEN Rapid TGE Ag Test Kit, Catalogue No. RG14-02; and ANIGEN Rapid Rota Ag Test Kit, Catalogue No. RG18-03; Animal Genetics Inc.; South Korea) according to the manufacture's protocol. Briefly, the diarrheal disease Ag cassette test is a chromatographic immunoassay for qualitative detection of virus antigen in porcine samples. The test cassette has the letter of "T" and "C" as a test line and a control line on the surface of the cassette. Both the test line and the control line in the result's window are not visible before application of the samples. The control line should always appear if the test procedure is carried out correctly and the test reagents of the control line function adequately. The purple test line will be visible in the result's window if there is sufficient amount of antigen in the specimen.

2.6. Experimental TGEV infection

Four 2-day-old piglets were used in this experiment; one of the piglets was used as a control. Three piglets were inoculated orally with 5 mL of the cell culture supernatant containing TGEV TMK22 strain (Federal Governmental Institute, All Russia Research Institute for Animal Health Microorganism Strain Collection), at a titer of 10^4 $TCID_{50}$/mL. The three piglets were killed just after the development of diarrhea (24 h post-infection); the control piglet was also killed. Intestine and fecal samples were collected and stored at −80 °C until use or used immediately.

2.7. Nucleotide sequence analysis

The analysis, alignment of nucleotide sequences, and the choice of the primers were conducted using the program package BioEdit version 7.0.5.2 (Hall, 1999) and Clustal W (Thompson et al., 1994). The evolutionary history of the sequences was inferred using the UPGMA method. The tree was drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All the positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

2.8. PCR products sequencing

DNA sequencing was carried out in the Federal Governmental Institute, All Russia Research Institute for Animal Health (FGI ARRIAH) research institute using automated DNA sequencer ABI Prism 3100.

2.9. Phylogenetic analysis

The nucleotide sequences of PEDV and PRV-A isolates obtained in this study are compared with the corresponding sequences of other virus strains, available in GenBank database using the computer program MEGA version 4.0 (Tamura et al., 2007).

3. Results

3.1. Development of virus-specific primers and RT-PCR

The sequence design of primers is the factor governing the sensitivity and specificity of RT-PCR assays.

Porcine respiratory coronavirus (PRCV) is a deletion mutant of TGEV in the S gene (Laude et al., 1993). Unlike TGEV, PRCV causes a respiratory tract infection with clinical or sub-clinical (pathological changes in the respiratory tract without any clear clinical signs) manifestations, with minimal or no intestinal replication. As a result of the high homology between the two virus genome sequences, the deletion region of the S gene of TGEV, which is absent in the PRCV genome, was targeted in this study. The M gene was used as a suitable target for the development of specific primers

Table 1
Primers used in multiplex nested RT-PCR.

Organism	Primers	Sequence (5′ → 3″)	Product length (bp)	Position
Porcine transmissible gastroenteritis virus (TGEV)	S1 (forward)	AGGGTAAGTTGCTCATTAGAAATAATGGTAAGTT	950	20,298–20,331[a]
	S2 (reward)	CTAATTTACCACTAACCAACGTGGAGCTATTA		21,216–21,247[a]
	S3 (forward)	AAAAATTATTTGTGGTTTTGGTTGTAATGCC	792	20,369–20,399[a]
	S4 (reward)	GTGTAGTAAAAACATTAGCCACATAACTAGCACA		21,127–21,160[a]
Porcine Epidemic Diarrhea Virus (PEDV)	M1 (Forward)	GAATTTTACATGGAATATCATACTGACGATACTACTTGT	450	25,748–25,773[b]
	M2 (Reward)	CGCCAGTAGCAACCTTATAGCCCTCTA		26,159–26,133[b]
	M3 (Forward)	TGCTTCAGTATGGCCATTACAAGTACTCTG	291	25,776–25,805[b]
	M4 (Reward)	CCTGTCGGCCCATCACAGAAGTAGT		26,066–26,042[b]
Porcine Group A Rotavirus (PRV-A)	P1 (Forward)	GGCTTTTAAAGCGCTACAGTGATGTCTCT	317	1–29[c]
	P2 (Reward)	GGTCGTGATTGTGTTGATGAATCCATAGA		289–317[c]
	P3 (Forward)	CTCAGCATTGACGTAACGAGTCTTCC	208	28–53[c]
	P4 (Reward)	TGAGTGGATCGTTTGAAGCAGAATCAGA		208–235[c]

[a] TGEV Purdue strain (accession no. NC_002306).
[b] PEDV strain CV777 (accession no. NC_003436).
[c] NSP5 gene of PRV-A OSU strain (accession no. X15519).

for the detection of PEDV. The nucleotide sequences of all the eleven genome segments of PRV-A were analyzed. The NSP5 gene, which is the most conservative gene among PRV-A strains and isolates, was therefore used for the development of group-specific primers.

Based on the sequence data generated, and the results of BLAST analysis, six primers for each target virus were designed and synthesized. These primers were used in the RT-PCR in different combinations. The nucleotide sequences of the primers used for the multiplex nested RT-PCR, and the expected size of the amplified DNA fragments are shown in Table 1.

The combinations which gave optimal results, the thermal conditions, $MgCl_2$ concentration, and the annealing temperatures were optimized. The optimal results (maximum band intensity and minimal background nonspecific staining) were obtained using the following RT-PCR procedures: for reverse transcription, 3 μL of the extracted RNA was denatured by heating to 85 °C for 2 min and immediately placed on ice for 2–3 min. To this RNA solution, a mixture of reagents consisting of 5 μL of AMV 5× buffer (Promega, Washington, USA) (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$); 1 mM of each deoxyribonucleotide triphosphate (dNTP) (Fermentas, Vilnius, Lithuania); 10 μM of each primer S1, S2, P1, P2, M1, and M2; 1.25 U of AMV reverse transcriptase (Promega, Washington, USA); and bidistilled water was added to make up a total volume of 25 μL. The mixture was incubated at 42 °C for 40 min. The cDNA (DNA copy) was either stored at −20 °C or amplified immediately. Two PCR assays were carried out. The first PCR (cDNA amplification) was carried out using primers S1, S2, P1, P2, M1, and M2. The second PCR (re-amplification step) was a nested PCR and conducted using the internal primers S3, S4, P3, P4, M3, and M4 (Sintol, Moscow, Russia). The PCR mixture contained 2.5 μL of 10× Taq DNA polymerase buffer (SibEnzyme Ltd, Novosibirsk, Russia); 3 mM of $MgCl_2$; 200 μM each of dGTP, dATP, dTTP, and dCTP; 5 μM of each primer; and 1.25 μL of Taq DNA polymerase (SibEnzyme Ltd, Novosibirsk, Russia). Deionized water was added to make up a total volume of 25 μL. The amplification step was carried out using the following thermal cycles: the denaturation step at 94 °C for 4 min was followed by 40 cycles of the amplification step (denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min), a final extension step at 72 °C for 5 min, and then the tubes were held at +4 °C. The product of the first reaction (2 μL) was used as a template for the reaction of the re-amplification, which was carried out using the following thermal cycles: the denaturation step at 94 °C for 4 min followed by 30 cycles of the amplification step (denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min), a final extension step at 72 °C for 5 min. PCR products were resolved by gel electrophoresis in 2% agarose gel in TBE (Tris–boric acid–EDTA) buffer, stained by ethidium bromide (Sigma, NY, USA). The product of the RT-PCRR was visualized using a UV transilluminator. Gel images were recorded digitally with the Gel Doc 2000 system (Bio-Rad Laboratories, NY, USA) (Fig. 1). The size of the amplified products (in the second reaction) was 792 bp, 291 bp, and 208 bp for TGEV, PEDV, and PRV-A, respectively.

The multiplex RT-PCR assay was standardized by testing the positive controls for the three viruses (PEDV, TGEV, and PRV-A) in three ways: RT-PCR mixture containing one primer pair and three templates, three primer pairs and one template, and three primer pairs and all the three templates. All RT-PCR products were identified by molecular sequencing (data not shown).

To compare the analytical sensitivity of the multiplex nested RT-PCR (three primer pairs and one template) versus conventional nested RT-PCR (one primer pair and one template), 10-fold serial dilutions of cell culture derived TGEV and PRV-A were carried out. For conventional nested RT-PCR for the detection of PEDV, TGEV,

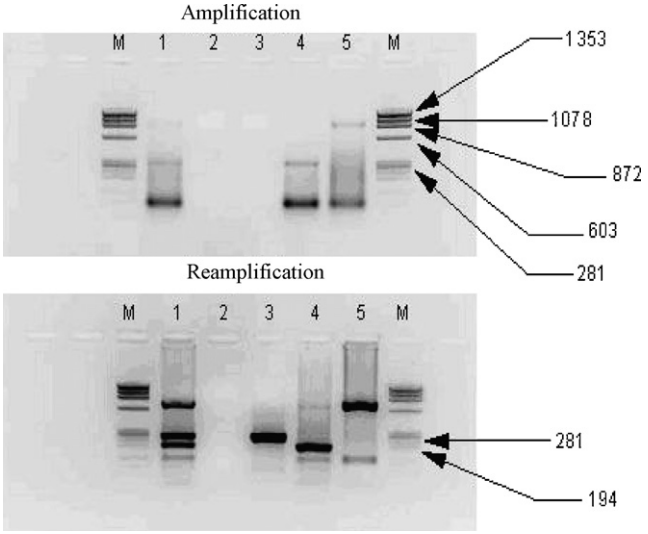

Fig. 1. Electrophoretic profile of multiplex nested RT-PCR products. M, DNA Ladder φX174/HaeIII. Lane 1, TGEV, TMK22 strain; PEDV isolate; and PRV-A, KR-47 strain. Lane 2, negative control (MilliQ water). Lane 3, PEDV isolate. Lane 4, PRV-A, KR-47 strain. Lane 5, TGEV, TMK22 strain.

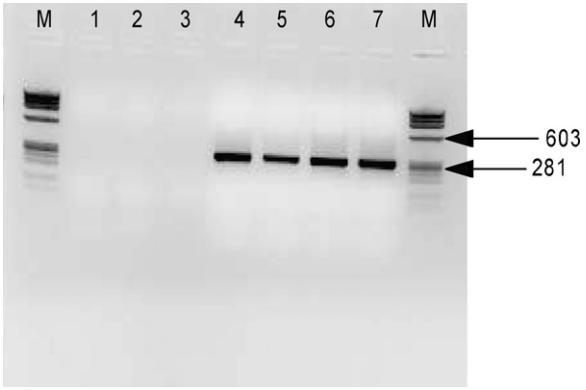

Fig. 2. Electrophoretic profile of the multiplex nested RT-PCR product for PEDV Moscow/07/05 isolated from porcine stool sample and inoculated into Vero cell culture. M, DNA marker φX174/HaeIII. Lane 1, 11th passage, lane 2, 10th passage; lane 3, 9th passage; lane 4, 8th passage; lane 5, 7th passage; lane 6, 6th passage; and lane 7, 5th passage.

and PRV-A, the same three sets of specific primers were used in separate tubes. The same volumes and concentration of the primers, reagents, and thermal cycler conditions for the multiplex RT-PCR were used.

Numerous attempts to cultivate PEDV isolates did not show any CPE in Vero cell culture; however the genome of PEDV isolate Moscow/07/05 was detected using the nested RT-PCR from the first up to the eighth blind passage (Fig. 2). The quantification of PEDV isolates was overcome in this study with the use of a spectrophotometer for the RNA quantification. To determine the detection limit of the nested RT-PCR assay, 10-fold serial dilutions of PEDV isolate from the first passage were used. After the RNA extraction and DNase treatment in RNase free microcentrifuge tube, the absorbance of the diluted RNA sample was measured at 260 and 280 nm with a dilution factor 10. The concentration of the nucleic acid is calculated using the Beer–Lambert law, which predicts a linear change in absorbance with concentration (RNA OD = 1 ≧ 40 μg/mL extinction coefficient = 0.025). The multiplex nested RT-PCR was able to detect PEDV RNA up to concentration of 27.2 μg/μL (OD_{260} = 68 and OD_{280} = 40) and 33.4 μg/μL by the conventional nested RT-PCR. The detection limit of the multiplex nested RT-PCR was 10^4 and 10^3 $TCID_{50}$/mL (after the first round PCR), 10^2 and 10^1 $TCID_{50}$/mL (after the second round PCR) for TGEV and PRV-A, respectively. The amplified product for PEDV was not detected in the first round PCR. The same results were obtained by conventional nested RT-PCR (data not shown).

3.2. Data analysis

Among the eighty-three animals, sixty-six were aged from 5 to 30 days and seventeen were above 30 days old. Among the 175 field samples collected from animals during the outbreaks of severe diarrheal disease, 87 (49.7%) (n = 49) contained at least one virus, whereas no virus was detected in the 88 remaining samples (50.3%) (n = 34). The PRV-A genome was detected in 64 samples (36.5%) (n = 34) and PEDV genome was detected in 23 samples (13%) (n = 21). Dual infection with PRV-A and PEDV was detected in seven samples (4%) (n = 6). The TGEV genome was not detected in any of the field samples. These results were shown in Table 2. The results obtained for TGEV were excluded from the analysis. The PEDV genome was detected mainly in the fecal samples (18, 27.7%) from eighteen animals in the acute stage of the infection. The PRV-A genome was detected in all pathological samples with predominance detection rate in fecal samples (27, 41.5%).

Table 2
Detection of viruses in different sample type.

Samples	No. of positive samples/total no. of samples (%) with:			P^a
	PEDV	PRV-A	Dual infection	
Feces	18/65 (27.7)	27/65(41.5)	3/65 (04.6)	<0.001
Intestines	5/55 (09.0)	19/55 (34.5)	4/55 (07.2)	<0.001
Lung/spleen	0/55 (00.0)	18/55 (32.7)	0/55 (00.0)	<0.001

[a] A P-value of ≤0.05 (two-sided χ^2 test) was considered significant for differences among PRV-A and PEDV.

3.3. Chromatographic immunoassay

All field samples collected were analyzed by the chromatographic immunoassay for the detection of TGEV, PEDV, and PRV-A. Among 175 samples, 87 were positive and 88 were negative. As shown in Table 3, the same results were obtained by the chromatographic immunoassay and the multiplex nested RT-PCR. Three lung/spleen samples were positive for PRV-A by the multiplex nested RT-PCR, yet negative by the chromatographic immunoassay. The dual infection with PEDV and PRV-A was detected in seven specimens by the multiplex nested RT-PCR and the chromatographic immunoassay. Dual infection with PEDV and PRV-A was detected in previous studies (Song et al., 2006).

The intestinal and fecal samples, taken from the three infected and the one control piglet, were analyzed by the multiplex nested RT-PCR. The six intestinal and fecal samples were positive for TGEV either by the nested RT-PCR or by the chromatographic immunoassay. The control piglet was negative for any of the three viruses using the two tests.

3.4. Nucleotide sequencing and phylogenetic analysis

Fifteen PRV-A isolates were selected and their nucleotide sequences were deposited in GenBank (accession nos. GQ907005, GQ907006, GQ907007, GQ907008, GQ907009, GQ907010, GQ907011, GQ907012, GQ907013, GQ907014, GQ907015, GQ907016, GQ907017, GQ907018, and GQ907019). Twelve PEDV isolates were selected for phylogenetic analysis. The obtained nucleotide sequences of PEDV isolates were compared with the nucleotide sequences of the type strain CV777 (NCBI accession number NC_003436) and seventeen PEDV strains available from the international GenBank (accession numbers: AF015888, AY608890, AY653205, AY974335, D89752, DQ845249, EF185992, EU031893, EU302820, EU287429, FJ196192, FJ196191, FJ196190, FJ196185, FJ196184, FJ196183, and FJ196182). The nucleotide sequences of PEDV isolates obtained in this study were deposited in GenBank under accession numbers: EU167541, and EU179721–EU179731.

Sequence analysis of the twelve PEDV isolates in Russia has shown nucleotide substitutions in three positions: 144 (G by A), 207 (T by C), and 303 (G by T) of the M gene. These nucleotide substitutions did not affect the amino acid composition of the M protein. Further molecular studies need to be done to study if these isolates undergo any other substitutions in the M gene or elsewhere in the PEDV genome. The nucleotide sequence analysis showed

Table 3
Comparative study of nested RT-PCR and Chromatographic Immunoassay (CIA) results for 175 piglet samples.

	Viral pathogens								
	PRV-A			PEDV			Dual infection		
	I/	F/	/L–S	I/	F/	/L–S	I/	F/	L–S
Nested RT-PCR	19	27	18	5	18	0	4	3	0
CIA	19	27	15	5	18	0	4	3	0

I: intestine, F: feces, L–S: lung and spleen.

that seven Thai isolates from two different regions in Thailand (FJ196192, FJ196191, FJ196190, FJ196185, FJ196184, FJ196183, and FJ196182) undergo nucleotide substitutions in this region of the M gene (Fig. 3).

As shown in Fig. 4, the thirty PEDV isolates were divided into two clusters A and B. Group A comprised of all Russian isolates, LZC, CV777, two Korean strains (KPEDV-9 and Chinju99), one Japanese strain (Jme2), and three Chinese isolates (HNXYYYP-2007, YM 2007, and DX). Group B comprised of all Tai isolates and three Chinese PEDV strains (JS-2004-2, LJB/03, and QH).

4. Discussion

The multiplex nested RT-PCR which was developed in this study was sensitive and was able to detect TGEV, PEDV, and PRV with high specificity as shown by molecular sequencing of the amplified

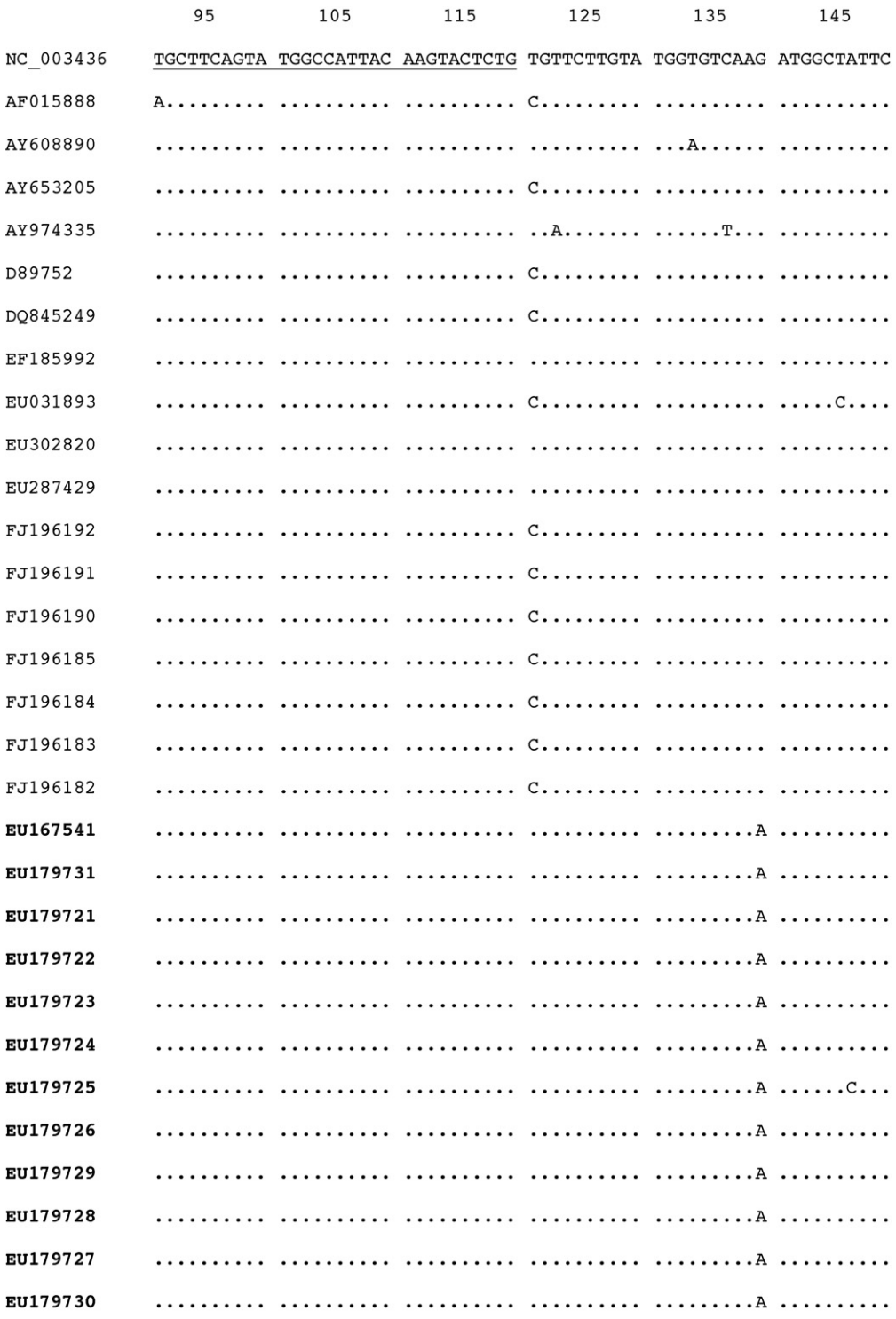

Fig. 3. Comparison of the nucleotide sequences of the amplified PEDV M gene region. The positions of specific primers (M3: forward primer, M4: reverse primer) are underlined. Russian isolates are in bold.

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                155         165         175         185         195         205
NC_003436       TATGGATACT TTGGCCTCTT GTGTTGGCAC TGTCACTTTT TGACGCATGG GCTAGCTTCC
AF015888        .......... .......... .......... .......... .......... ..........
AY608890        .......... .......... ....C..... .......C.. .......... ........T.
AY653205        .......... .......... .......... .......... ...T...... ........T.
AY974335        .......... .......... .......... .......C.. .......... ........T.
D89752          .......... .......... .......... .......... .......... ..........
DQ845249        .......... .......... .......... .......... .......... ..........
EF185992        .......... .......... .......... .......... ........C. C.........
EU031893        .......... .......... .......... .......... ...T...... ..........
EU302820        .......... .......... .......... .......C.. ...T...... ..........
EU287429        .......... .......... .......... .......... ...T...... ..........
FJ196192        .......... .......... .....A.... .......... ...T...... ........T.
FJ196191        .......... .......... .....A.... .......... ...T...... ........T.
FJ196190        .......... .......... .....A.... .......... ...T...... ........T.
FJ196185        .......... .......... .....A.... .......... ...T...... ........T.
FJ196184        .......... .......... .....A.... .......... ...T...... ........T.
FJ196183        .......... .......... .....A.... .......... ...T...... ........T.
FJ196182        .......... .......... .....A.... .......... ...T...... ........T.
EU167541        .......... .......... .......... .......... .......... ..C...C...
EU179731        .......... .......... .......... .......... .......... ..C...C...
EU179721        .......... .......... .......... .......... .......... ..C...C...
EU179722        .......... .......... .......... .......... .......... ..C...C...
EU179723        .......... .......... .......... .......... .......... ..C.......
EU179724        .......... .......... .......... .......... .......... ..C.......
EU179725        .......... .......... .......... .......... .......... ..C.......
EU179726        .......... .......... .......... .......... .......... ..C.......
EU179729        .......... .......... .......... .......... .......... ..C.......
EU179728        .......... .......... .......... .......... .......... ..C.......
EU179727        .......... .......... .......... .......... .......... ..C.......
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Fig. 3. (*Continued*)

products. The multiplex nested RT-PCR was applied in this study to facilitate surveillance of PEDV replication in cell culture. The use of an immunochromatographic test strip such as the Anigen Rapid Virus Ag Test kit (ANIGEN Rapid PED Ag Test Kit, Catalogue No. RG14-01; ANIGEN Rapid TGE Ag Test Kit, Catalogue No. RG14-02; and ANIGEN Rapid Rota Ag Test Kit, Catalogue No. RG18-03; Animal Genetics Inc.; South Korea) can be used as a rapid method for the detection of gastroenteritis infections in farms and in the laboratory but the results need to be confirmed and characterized further by RT-PCR and other available methods depending on the laboratory facilities. Nucleotide sequencing of the amplified fragment of the PEDV M gene showed three nucleotide substitutions. Phylogenetic analysis classified the PEDV isolates detected in one cluster (cluster A). According to the results of this study these substitutions can be considered as biomarkers for Russian PEDV isolates but this approach needs to be investigated further. Coronaviruses adapt rapidly to changing ecological circumstances by the high mutation rate of their RNA genome (about 10^4 nucleotide substitution/site/year) and the high recombination frequencies (Sanchez et al., 1992). The genetic variability and the emergence of new

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EU179730     ..C....... .......... .......... .......... .......... ..C.......

                215        225        235        245        255        265
NC_003436    AGGTCAACTG GGTCTTTTTC GCTTTCAGCA TCCTTATGGC TTGCATCACT CTTATGCTGT
AF015888     .......... .......... .......... .......C.. .......... ..........
AY608890     .......... .....C.... .......... .......... .......... ....C.....
AY653205     .......... .........T .......... .......... .......... ..........
AY974335     .......... .......... .......... .......... .......... ..........
D89752       .......... .......... .......... .......... .......... ..........
DQ845249     .......... .......... .......... .......... .......... ..........
EF185992     .......... .......... .......... .......... .......... ..........
EU031893     .......... .......... .......... .......... .........C ..........
EU302820     .......... .......... .......... .......... .......... ..........
EU287429     .......... .......... .......... .......... .......... ..........
FJ196192     .......T.. .........T .......... .......... .......... ..........
FJ196191     .......T.. .........T .......... .......... .......... ..........
FJ196190     .......T.. .........T .......... .......... .......... ..........
FJ196185     .......T.. .........T .......... .......... .......... ..........
FJ196184     .......T.. .........T .......... .......... .......... ..........
FJ196183     .......T.. .........T .......... .......... .......... ..........
FJ196182     .......T.. .........T .......... .......... .......... ..........
EU167541     ........C. ......C... ..C....... .......... .......... ..........
EU179731     ........C. ......C... ..C....... .......... .......... ..........
EU179721     ........C. ......C... ..C....... .......... .......... ..........
EU179722     ........C. ......C... ..C....... .......... .......... ..........
EU179723     .......... .......... .......... .......... .......... ..........
EU179724     .......... .......... .......... .......... .......... ..........
EU179725     .......... .......... .......... .......... .......... ..........
EU179726     .......... .......... .......... .......... .......... ..........
EU179729     .......... .......... .......... .......... .......... ..........
EU179728     .......... .......... .......... .......... .......... ..........
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Fig. 3. (*Continued*)

coronavirus strains which can infect new host species and cause emerging diseases with or without possible zoonotic risk are not rare phenomena as was shown for other human and animal coronaviruses (TGEV and the attenuated strain PRCV, severe acute respiratory syndrome (SARS), human coronavirus OC43, and bovine coronavirus) (Vijgen et al., 2005). In addition, the genomes of canine and feline coronaviruses can recombine *in vivo* and develop into different biotypes that are transmissible serially in a new host species (Herrewegh et al., 1998).

The opportunity of host-jumping events is also given for other animal RNA-viruses and PEDV is not excluded from this group of viruses. For this reason, the detection and the molecular analysis of new emerging strains play a key role in the surveillance and the elucidation of viral and host factors which may contribute and facilitate interspecies genetic transfer. The use of specific laboratory methods for the detection and the characterization of porcine enteric viruses such as RT-PCR and chromatographic immunoassay could contribute to such mission.

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EU179727    .......... .......... .......... .......... .......... ..........
EU179730    .......... .......... .......... .......... .......... ..........

                275        285        295        305        315        325
NC_003436   GGATAATGTA TTTTGTCAAT AGCATTCGGT TGTGGCGCAG GACACATTCT TGGTGGTCTT
AF015888    .......... .......... .......... .......... .......... ..........
AY608890    .......... C......... .......... .......... .......... ..........
AY653205    .......... C......... .......... .......... .......... ..........
AY974335    .......... C......... .......... .......... .......... .........C.
D89752      .......... .......... .......... .......... .......... ..........
DQ845249    .......... .......... .......... .......... .......... ..........
EF185992    .......... .......... .......... .......... .......... ..........
EU031893    .......... .......... .......... .......... .......... ..........
EU302820    .......... .......... .......... .......... .......... ..........
EU287429    .......... .......... .......... .......... .......... ..........
FJ196192    .......... C......... .......... .......... .......... ..........
FJ196191    .......... C......... .......... .......... .......... ..........
FJ196190    .......... C......... .......... .......... .......... ..........
FJ196185    .......... C......... .......... .......... .......... ..........
FJ196184    .......... C......... .......... .......... .......... ..........
FJ196183    .......... C......... .......... .......... .......... ..........
FJ196182    .......... C......... .......... .......... .......... ..........
EU167541    .......... .C........ ........T. .......... .........C ..........
EU179731    .......... .C........ ........T. .......... .........C ..........
EU179721    .......... .C........ ........T. .......... .........C ..........
EU179722    .......... .C........ ........T. .......... .........C ..........
EU179723    .......... .......... ........T. .......... .......... ..........
EU179724    .......... .......... ........T. .......... .......... ..........
EU179725    .......... .......... ........T. .......... .......... ..........
EU179726    .......... .......... ........T. .......... .........C ..........
EU179729    .......... .......... ........T. .......... .........C ..........
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Fig. 3. (*Continued*)

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EU179728    .......... .......... ........T. .......... .........C ..........
EU179727    .......... .......... ........T. .......... .........C ..........
EU179730    .......... .......... ........T. .......... .......... ..........
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                335        345        355        365        375
NC_003436   TCAATCCTGA AACTGACGCG CTTCTCACTA CTTCTGTGAT GGGCCGACAG G
AF015888    .......... .......... .......... .......... .......... .
AY608890    .......... ...A...... .......... .......... .......... .
AY653205    .......... ...A...... .......... .......... .......... .
AY974335    .......... ...A...... .......... .......... .......... .
D89752      .......... .......... .......... .......... .......... .
DQ845249    .......... .......... .......... .......... .......... .
EF185992    .......... .......... .......... .......... .......... .
EU031893    .......... .......... .......... .......... .......... .
EU302820    .......... .......... .......... .......... .......... .
EU287429    .......... .......... ....C..... .......... .......... .
FJ196192    .......... ...A...... .......... .......... .......... .
FJ196191    .......... ...A...... .......... .......... .......... .
FJ196190    .......... ...A...... .......... .......... .......... .
FJ196185    .......... ...A...... .......... .......... .......... .
FJ196184    .......... ...A...... .......... .......... .......... .
FJ196183    .......... ...A...... .......... .......... .......... .
FJ196182    .......... ...A...... .......... .......... .......... .
EU167541    .......... .......... .......... .......... .......... .
EU179731    .......... .......... .......... .......... .......... .
EU179721    .......... .......... .......... .......... .......... .
EU179722    .......... .......... .......... .......... .......... .
EU179723    .......... .......... .......... .......... .......... .
EU179724    .......... .......... .......... .......... .......... .
EU179725    .......... .......... .......... .......... .......... .
EU179726    .......... .......... .......... .......... .......... .

EU179729    .......... .......... .......... .......... .......... .
EU179728    .......... .......... .......... .......... .......... .
EU179727    .......... .......... .......... .......... .......... .
EU179730    .......... .......... .......... .......... .......... .
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Fig. 3. (*Continued*).

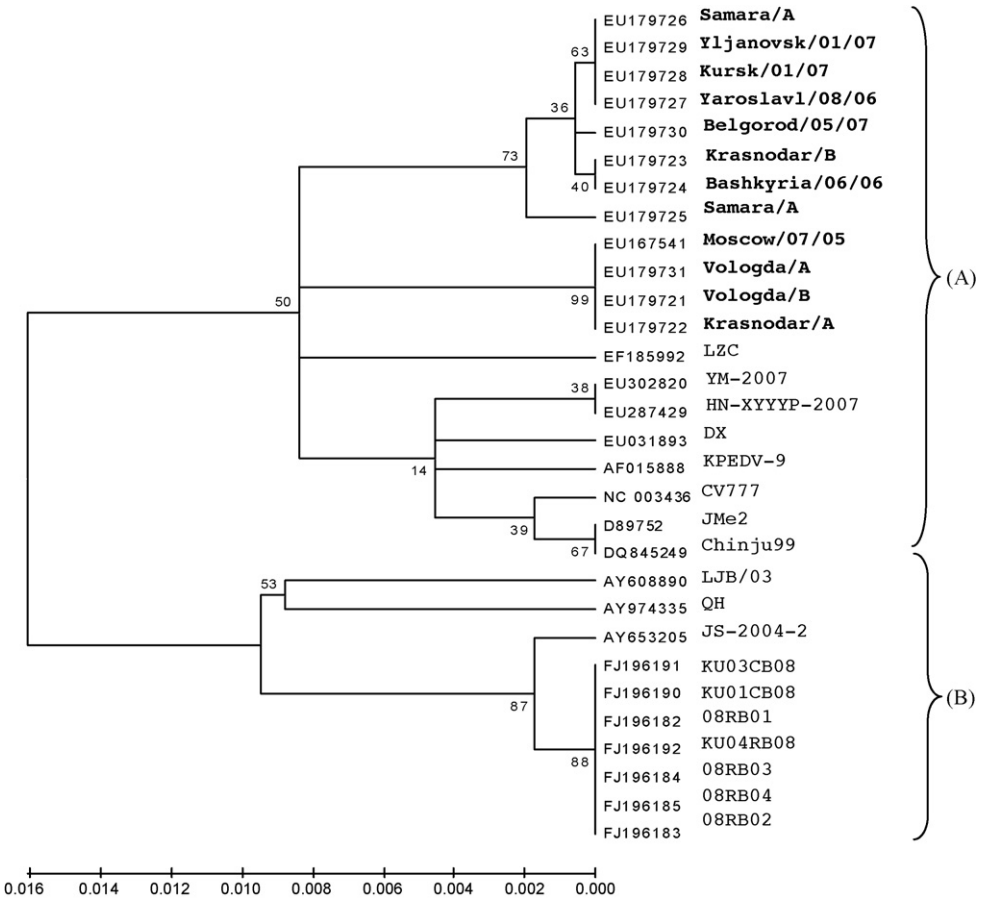

Fig. 4. Evolutionary relationships of PEDV Russian isolates and published strains based on the expected fragment comparison. (A) Cluster A and (B) cluster B. NCBI accession numbers, strain and isolate designations are given. Russian isolates are in bold.

Acknowledgment

Thanks to Pr. Z. Baybikov Tayfik (Laboratory of Porcine Diseases, FGI ARRIAH, Vladimir, Russia) for his encouragement and support during this study.

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