Arch Virol (2006) 151: 1525–1538
DOI 10.1007/s00705-006-0731-1

Archives of Virology
Printed in Austria

Detection and identification of avian, duck, and goose reoviruses by RT-PCR: goose and duck reoviruses are part of the same genogroup in the genus *Orthoreovirus*

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Received March 16, 2005; accepted January 19, 2006
Published online February 26, 2006

Summary. A reverse transcription-polymerase chain reaction (RT-PCR) procedure for the detection of avian, duck, and goose reovirus (ARV, DRV, and GRV) RNA from cell culture supernatant and clinical samples was established. Based on multiple sequence alignment, a pair of degenerate primers was selected and synthesized. The amplified, cloned, and sequenced 598-base-pair products from the σA-encoding gene fragment from 16 isolates (ranging over 30 years) indicated that the primer regions were well conserved. The sensitivity of this method was determined to be 10^{-2} PFU. The specificity of the RT-PCR method was determined by testing specimens containing avian influenza A viruses, Newcastle disease virus, and infectious bronchitis virus, all of which yielded negative results with no discernible background. The efficiency of the system for detection of ARV, DRV, and GRV directly in 71/83 clinical samples was confirmed. The nucleotide sequence analysis indicated that DRV and GRV isolated from China in different locales and years were closely related, showing 97.4–100% homology to each other, but with only 86.7–88.5% identity to DRV 89026. The nucleotide and amino acid sequence identities in the amplified σA-encoding gene were 74.2–78.4% and 86.9–92.0%, respectively, between duck/goose and chicken species. Phylogenetic analysis indicated that GRV and DRV aggregated into the same specified genogroup within subgroup II of the genus *Orthoreovirus* and are more closely related to ARV than to Nelson Bay virus. Overall, this study developed a sensitive and specific technique for the identification ARV, DRV, and GRV, and sequencing analysis has enhanced our understanding of the evolutionary relationship between ARV, DRV, and GRV.

Introduction

The genus *Orthoreovirus* of the family *Reoviridae* consists of four species that separate into three distinct subgroups [33]: Subgroup I includes the nonfusogenic mammalian othoreoviruses (MRVs), subgroup II includes the fusogenic avian reoviruses (ARVs) and Nelson Bay virus (NBV), and subgroup III is represented by the baboon reovirus (BRV). In spite of being from a mammalian host, NBV showed approximately 30 to 60% amino acid sequence identity to ARV, which is well below the greater than 90% identity exhibited by members within the ARV and MRV groups [5]. Duck reovirus is a tentative species assigned to the genus *Orthoreovirus* in the latest report of the International Committee on Taxonomy of Viruses (ICTV). Despite common properties shared between duck and chicken reovirus (named avian reovirus), the two viruses are antigenically different [10, 14] and their σC-encoding genes showed only 21–25% identity at nucleotide and amino acid levels [14, 36].

Although some of the duck and goose strains described so far seemed to be non-pathogenic in nature, some strains have been reported to cause diseases and even death [8, 10, 12, 21, 26–28]. Highly pathogenic Muscovy duck reovirus yl/79 has been confirmed to induce severe disease and death by pathogenicity tests [10]. More recently, Muscovy duck isolates have been reported to cause high morbidity and over 10% mortality [11, 16]. In infected duckling and goslings, clinical signs first appear usually in the second week after hatching; these are normally apathy accompanied by diarrhoea and reluctance to move. Dead birds can show macroscopically fibrinous pericarditis, friable liver, and marbled spleen [8, 21, 22, 25, 26]. Recovered ducks are markedly stunted in growth.

Despite the severity of diseases caused by duck and goose reovirus, very few attempts have been made to quickly diagnose the virus upon a disease outbreak or to confirm the identity of reovirus isolated during routine examinations. As these viruses have the potential to cause death in a wide variety of aquatic bird species (duck and goose), it is very important for the veterinary authorities to have a reliable diagnostic method to detect them. Furthermore, a sensitive and specific diagnostic method is essential if dissemination of the virus is to be controlled, as no vaccines currently are available for its prevention. Classical detection methods have involved virus propagation in reovirus-negative duck/goose/chicken embryo or embryo fibroblasts (DEFs/GEFs/CEFs) [4, 9] and detection via purification and electron microscopy, all which are laborious and time-consuming. Antigen detection assays vary widely in sensitivity and specificity. Diagnostic strategies based on detection of avian and mammalian reovirus RNA have been described [2, 6, 7, 15, 18, 20, 30, 32], but these methods have been shown to be incapable of detecting duck reovirus. Until now, diagnostic methods for duck and goose reovirus RNA detection have not been described. The development of rapid, simple, sensitive, and broad-spectrum avian reovirus detection from cell culture and clinical samples based on RT-PCR are required.

The S1 gene of DRV and the S2 gene segment of ARV encode the same major core protein, σA [14, 19, 29]. The viral σA-encoding gene segment was chosen as the target for PCR amplification because the σA is the major core

protein and therefore likely to be conserved among other products of reovirus strains. This RT-PCR technique is sufficiently sensitive and specific to amplify σA-encoding gene segment from 16 independent DRV, GRV, and ARV strains and is robust enough to detect a wide range of field-isolate strains of reovirus. In addition, it could be very useful in contributing to the creation of a databank of circulating reovirus sequences and extend our knowledge of ARV, DRV, and GRV evolutionary relationships.

Materials and methods

Cell culture

Duck embryo fibroblasts (DEF), goose embryo fibroblasts (GEF), and chicken fibroblasts (CEF) were prepared as described previously [10].

Treatment of field-origin samples

Reovirus disease was diagnosed between the years 2003–2004 in chickens, ducks, and geese on seven farms that are geographically distributed from south to northeast China. The clinical signs in the acute phase included a general malaise accompanied by diarrhoea and difficulties in movement. For duck and geese flocks, the earliest onset of disease was at 7 days of age and the latest at about 7–8 weeks of age. The outbreaks lasted 2–4 weeks. Morbidity ranged from 10 to 60%, and mortality from 10 to 20%. Mortality was higher in 2–3-week-old young birds, and recovered birds were stunted in growth. Multiple disseminated, small, grayish-white foci were seen in the liver of ducks and geese. Eighty-three livers of chickens, ducks, and geese with signs of arthritis/tenosynovitis were collected from 7 different farms and stored at −70 °C. Liver samples were ground and homogenized at 1:2 (v/v) in cold phosphate-buffered saline (PBS), and solid debris was pelleted by centrifugation at 3,800 × g for 30 min. The supernatant was filtered through a sterile membrane filter with a 0.45 μm pore size and filtrates were used for RNA extraction or stored at −70 °C. A 200 μl volume of the filtrate was inoculated into DEF/GEF/CEF and cell cultures with CPE were harvested and tested for reovirus by direct immunofluorescence assay (DIF) or immunodiffusion gel assay according to standard procedures [1, 25, 31].

Viruses

The viruses listed in Table 1 were isolated from the livers of chickens, ducks, and geese with arthritis/tenosynovitis, plaque-purified, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [11, 14]. Avian reference strains S1133, 1733, 2408, 138, 176, and 601G were obtained from the Institute of Veterinary Drug Control, China.

Determination of virus titer

DEF, GEF, and CEF monolayers cultured in 60-mm petri dishes were inoculated with serial dilutions of virus suspension. After a one-hour adsorption period, monolayers were infected with BME containing 1% agar and 0.0075% neutral red. The petri dishes were incubated at 37 °C in 5% CO_2 until plaques appeared (usually 3–5 days).

Nucleic acid extractions

200 μl virus stock and filtrates of liver homogenates were used for RNA extraction. Nucleic acid was extracted with Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA)

Table 1. Reoviruses used in this study

Virus	Species	Origin	Date of isolate	GenBank accession No.	Reference
S1133	CK	US	1975	AF104311	Liu and Huang, 2001 [19]
1733	CK	US	1983	AF293773	Liu and Huang, 2001 [19]
138	CK	US	N	AF059717	Liu and Huang, 2001 [19]
176	CK	US	N	AF059716	Liu and Huang, 2001 [19]
2408	CK	US	N	AF247724	Liu and Huang, 2001 [19]
601G	CK	TW	1992	AF294770	Liu and Huang, 2001 [19]
99G	CK	CN (HN)	1999	AY962257	this study
17	CK	CN (FJ)	1989	AY962258	this study
DRVS14	MD	CN (FJ)	1997	AY962259	this study
DRV044	D	CN (HLJ)	2004	AY962260	this study
DRVF	D	CN (HN)	2004	AY962261	this study
DRVC4	MD	CN (FJ)	1998	AY962265	this study
GRV040	G	CN (HLJ)	2004	AY962262	this study
GRV042	G	CN (HLJ)	2004	AY962263	this study
GX	G	CN (GZ)	2000	AY962264	this study
WKG	G	CN (HLJ)	2003	AY962266	this study

according to the manufacturer's instructions. Nucleic acid extracts were tested by RT-PCR immediately or stored at −70 °C. To exclude laboratory contamination, RNA isolation and PCR mixture preparation were done in rooms where cloned and amplified reovirus nucleic acids had never been used. All handling was with different sets of pipettes and the exclusive use of filter tips. Each RT-PCR was screened routinely for contamination using negative reagent controls.

RT

RT was performed in accordance with the M-MLV manufacturer's (Invitrogen, Life Technologies, Carlsbad, CA) instructions. A 6 μl aliquot of nucleic acid was incubated at 94 °C for 2 min in the presence of 250 pM random hexamers and then snap cooled on ice. Twelve microliters of chilled RT mixture was added, resulting in a final concentration of 1 × RT buffer, 5 mM $MgCl_2$, 0.1 M DTT, and 1 mM dNTP (TaKaRa Biotechnology Co., Ltd.). Reaction mixtures were incubated at 42 °C for 2 h and then finally at 70 °C for 15 min. Each RT assay mixture included an RT control that contained water in place of nucleic acid.

Oligonucleotide primers

Two degenerate oligonucleotide primers (PAF and PAR) were designed based on the sequence alignment of ARV genome segment S2 from isolates S1133(AF104311), 1733(AF293773), 2408(AF247724), 176(AF059716), 138(AF059717), 601G(AF311322), T2-TW(AF294765), 918(AF294766), 1017(AF294762), and DRV genome segment S1 from isolates 89026(AJ278102), SY04(DQ013346), and CX04(DQ0013348), using the CLUSTAL W computer program (DNASTAR 6.0, Madison, WI, USA). The primer pair was selected based on conserved regions located within the major core protein gene. The primers were then used for RT-PCR amplification with PAF as the sense (upstream) primer and PAR as the antisense (downstream) primer. The PAF primer hybridizes to nucleotide position 34–55 of the σA-encoding gene, while the PAR primer hybridizes to nucleotide position 640–659.

PCR

PCR was performed in accordance with the manufacturer's instructions (Biometra Germany) in a T-Gradient Thermoblock. In the primary PCR, 3 μl of the RT reaction mixture was used in a 50 μl final reaction volume, with final concentrations of 1 × PCR buffer II, 2.5 mM $MgCl_2$, 500 μM each dNTP, 1 μM each oligonucleotide primer, and 1.25 U of DNA polymerase (TaKaRa Biotechnology Co., Ltd.). Samples were incubated at 94 °C for 5 min, and this was followed by 30 cycles each of 30 s denaturation at 94 °C, 30 s primer annealing at 53 °C and 1 min extension at 72 °C, and a final extension for 10 min at 72 °C.

Agarose gel electrophoresis

The PCR products were visualized by agarose gel electrophoresis. The gel contained 1 × Tris-acetate-EDTA, 1% agarose, and 200 μg of ethidium bromide per liter. Amplification products were visualized with UV light and photographed using a Thermal Imaging System FTI-500 (Pharmacia Biotech). The samples were scored positive for the reovirus σA-encoding gene segment if DNA fragments of the appropriate sizes were detected in the PCR product. The DNA segment corresponding in size to the gene segment of interest was extracted using an Agarose Gel DNA extraction kit (Watson Biotechnologies, Inc. Shanghai, China).

Sequencing and analyzing the σA-encoding gene

RT-PCR products were either sequenced directly or after cloning with a pMD18-T cloning kit (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer's instructions. Recombinant plasmids were used to transform *Escherichia coli* strain DH5 α competent cells. Recombinant plasmids containing the σA-encoding cDNA segment were cleaved with *Eco*RI/*Hin*dIII. Positive cultures were grown overnight, and plasmids were purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA). The purified products were sequenced commercially on a model 3730 DNA sequencer (DNA Sequence Service, TaKaRa Biotech Co). Splits of purified products obtained from duplicate clones of each sample were sequenced and analyzed to identify potential sequencing errors and PCR artifacts. The sequencing information was compiled initially with the Seqman program (DNASTAR, Madison, Wis.), and the nucleotide sequences were compared initially with the MegAlign program (DNASTAR).

Phylogenetic analysis of σA-encoding gene segment nucleotide sequences

Nucleotide sequences from the σA-encoding gene of ARV, DRV, GRV, NBV (GeneBank accession No. AF059718), mammalian reoviruses T1L(L19774), T2J(L19775), T3D(L19776), and BRV(AF059719) were used to create a phylogenetic tree for relationship studies. In the present study, the σA-encoding gene sequence corresponding to amino acid residues 14-212 was used to generate a phylogenetic tree.

Nucleotide sequence accession numbers

The nucleotide sequences obtained using the optimized RT-PCR assay were deposited in GenBank and their GenBank Numbers are shown in Table 1.

Results

Primer sequences

Phylogenetic analysis of σA-encoding genes showed that ARVs separated into two major genotypes [9]. To identify regions of maximum sequences conservation, the

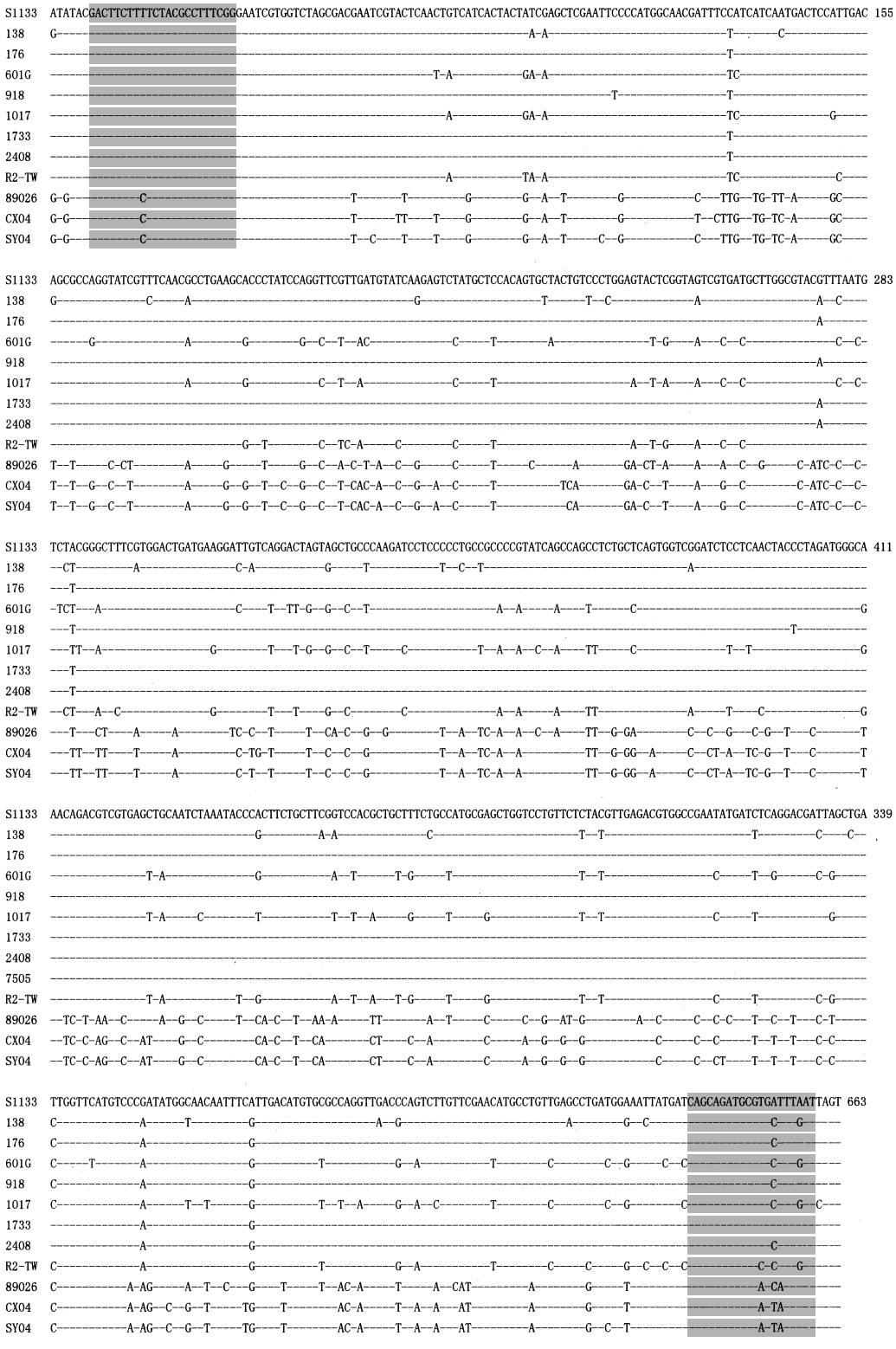

Fig. 1. Multiple alignments of the σA-encoding gene segment from avian and duck reovirus strains (nucleotides 28 to 663). Two conserved regions (shadow box), 34–55 and 640–659, were used for the design of primers for the RT-PCR assay

Table 2. Primers used for RT-PCR amplification

Designation	Nucleotide position (nt)	Sequence
PAF	34–55	5′-ACTTCTTY[a]TCTACGCCTTTCG-3′
PAR	640–659	5′-ATY[b]AAW[c]D[d]CW[e]CGCATCTGCTG-3′

[a]The 14th nt from the 3′ end of primer PAF
[b,c,d] and [e] are the 3rd, 6th, 7th, and 9th residues from the 5′ end of primer PAR

nucleotide sequences of nine ARVs from two different genotypes and three DRVs were aligned (Fig. 1). Despite being different species, the σA-encoding genes of DRV and ARV strains exhibited some degree of conservation in their nucleotide sequences. By this rationale, two sites of conserved regions, nucleotides 34–55 and 640–659 from the 5′ end of the σA-encoding gene were selected as PCR targets. Primers for RT-PCR were designed such that the sequence regions at the 3′ ends of the primers were absolutely conserved (13 and 11 nucleotides). Closer analysis of the sequences in the region 34–55 nucleotides from the 5′ end of the forward primer PAF indicated that 20 out of 21 nucleotides (nt) were conserved between ARV and DRV (Fig. 1). There was only one nucleotide mismatches found at the 14th nt from the 3′ end of primer PAF (Table 2). A downstream primer PAR is indicated in Table 2. In the region of nt 640–659 (Fig. 1), 16 out of 20 nt were common to all ARV and DRV species. The last 11 nt from the 3′ end of primer PAR exactly matched the nucleotide sequences present in all twelve strains of ARV and DRV. In order to allow the most sensitive detection of DRV and ARV, a degeneracy of the PAF primer was required: the Y residue was inserted at the 8th residue from the 5′ end of primer PAF. For the downstream primer PAR, Y, W, D, and W degeneracies were introduced, respectively, at the 3rd, 6th, 7th, and 9th residues from the 5′ end of primer (Table 2). The primers PAF and PAR designed here were mainly meant for the detection of DRV and ARV and were not intended for the detection of GRV due to the unavailability of GRV sequences. However, four GRV strains were tested using the RT-PCR assay described here and, surprisingly, a specific band was observed for these strains (data not shown).

Detection of the reovirus σA-encoding gene by RT-PCR

RT-PCR was carried out using reovirus dsRNA from different bird species (listed in Table 1) as templates. Use of the pair of primers (PAF and PAR) revealed that a single specific product of approximately 600 bp was detected (data not shown). The identities of these amplified σA-encoding gene segment PCR products were confirmed by nucleotide sequence analysis. The strains used here were collected from different host species and geographic locations over a period of thirty years. The S1 of DRV/GRV and S2 gene fragment of ARV were successfully amplified by using the optimized σA-encoding gene segment RT-PCR assay.

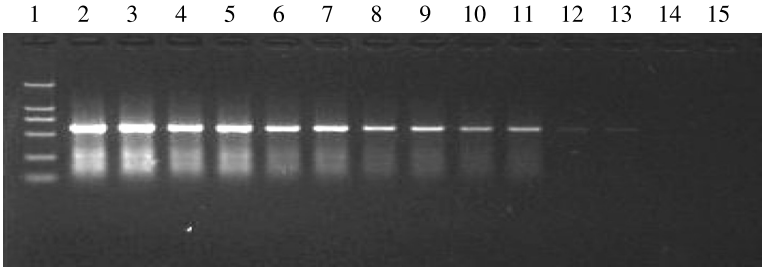

Fig. 2. Determination of RT-PCR detection sensitivity of duck reovirus S14. Lanes *2* to *15*, RT-PCR products obtained from 10^{-1} to 10^{-7} virus stock dilutions (in duplicate), respectively; *1*, DL2 000 DNA Marker. The lowest dilution of virus detected was the 10^{-6}

Sensitivity of the RT-PCR assay

To determine the sensitivity of primers for DRV, a RT-PCR was performed with 10-fold serial dilutions of the DRV S14 stock. DRV was amplified up to 10^{-6} (Fig. 2), which was equivalent to approximately 0.01 PFU per reaction mixture (data for ARV and GRV not shown).

Specificity of σA-encoding gene RT-PCR assay

To define the specificity of the newly described primers for ARV, DRV, and GRV, nucleic acids from viral isolates corresponding to a number of distinct virus families were extracted and utilized as templates in the σA-encoding gene RT-PCR assay. High-titer stocks of influenza virus, infectious bronchitis virus, and Newcastle disease virus were prepared from cell culture. Nucleic acids were extracted from these stocks and tested by using PAF and PAR primer pairs individually. In no case did the oligonucleotide primers amplify sequence fragments of the appropriate sizes (data not shown). Therefore, the newly described oligonucleotide primers might seem to possess a high degree of specificity for the ARV, DRV, and GRV σA-encoding gene segments.

Amplification of clinical samples by RT-PCR

Cell cultures that tested positive by DIF or immunodiffusion gel assay were all RT-PCR positive. For liver samples, which showed up as positive in cell culture DIF or immunodiffusion gel assay, 71 (85%) produced a 600-bp RT-PCR product.

Analysis of nucleotide and deduced amino acid sequences

To better understand genetic diversity among DRV, GRV, and ARV, 597 nucleotide and deduced amino acid (residues 14 to 212) sequences of the σA protein were aligned with homologous published genes (data not shown) and proteins (Fig. 3). ARV reference strains (S1133, 1733, 138, 176, 2408, 601G) were 100% homologous to the corresponding GenBank sequences. There were no apparent deletions or insertions in this 597-nucleotide sequence region (data not shown). The amino

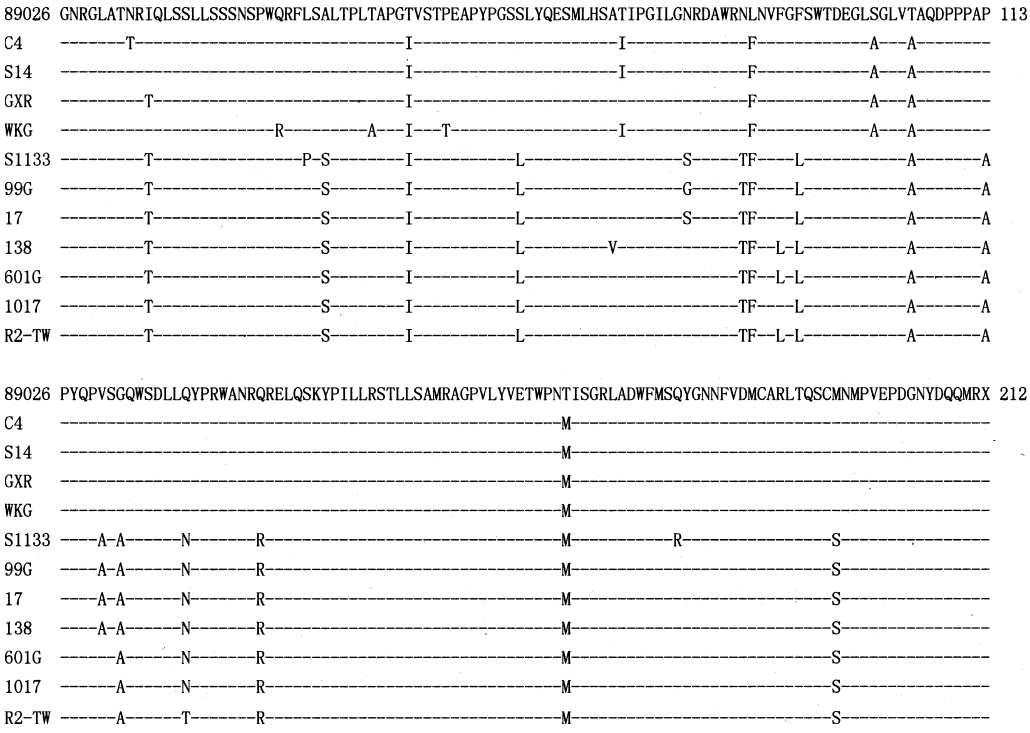

Fig. 3. Alignment of deduced amino acid sequences of σA (residues 14 to 212). DRV 89026 σA are shown in the single-letter code. Residues that are identical to the 89026 sequences are indicated by dashes. Amino acid positions are numbered right the sequences, Strains with identical amino acid sequences in this region of σA are (1) DRVS14, GRV040, DRV044, DRVF, and GRV042; (2) ARV 17, 176, 2408, and 1733

Table 3. Percentage identities of homologous σA-encoding gene and protein of DRV, GRV, ARV, and other orthoreoviruses

	C4/S14	89026	WKG	S1133	1733	138	916	NBV	BRV	MRV
C4/S14		96	99	88	90	89	91	60	28	31
89026	89		95	87	89	89	89	60	28	31
WKG	99	89		87	89	88	89	60	28	30
S1133	74	75	74		98	96	96	60	30	32
1733	75	75	75	99		97	97	60	30	32
138	75	76	75	93	94		97	60	30	32
916	77	78	77	87	87	86		61	30	32
NBV	59	59	58	61	60	60	60		32	32
BRV	42	43	41	44	43	44	44	43		28
MRV	42	40	42	42	43	41	42	41	40	

The percent identities were determined by pairwise comparisons of the homologous σA-encoding gene segments of the various orthoreoviruses and their encoded proteins. The numbers in the top right of the table indicate amino acid identities; whereas the bottom left numbers indicate nucleotide identities

acid sequence region from residues 136 to 212 is highly conserved among these different bird species viruses (Fig. 3). An analysis of the nucleotide sequences indicates that all of the duck and goose reoviruses isolated from China in different locales and years were closely related, showing 97.4–100% homology to each other, but they were quite different from French DRV-89026, with 86.7–88.5% identity (Table 3). The ARV99G and ARV17 isolates from China showed the highest homology to American ARV176, with 99.5% and 99.8%, respectively (data not shown). Comparisons between DRV/GRV and ARV revealed 74.2–78.4% nucleotide and 86.9–91.5% amino acid sequence identity. The DRV/GRV σA-encoding gene segments shared only about 60% identity to the NBV major core protein. By contrast, sequence comparisons of these DRV/GRV σA proteins with MRV (T1L, T2J, and T3D) or BRV homologues revealed extensive divergence, with amino acid identities that ranged from 28 to 42% (Table 3).

Phylogenetic analysis of the σA-encoding gene segment

Evolutionary relationships among σA-encoding genes of DRV, GRV, and ARV were determined by phylogenetic analysis. The σA-encoding gene of DRV/GRV and ARV segregated into different groups (Fig. 4). This result supported the classification of GRV and DRV into the previously defined subgroup II in the genus *Orthoreovirus*, which includes ARV and NBV. The extent of conservation

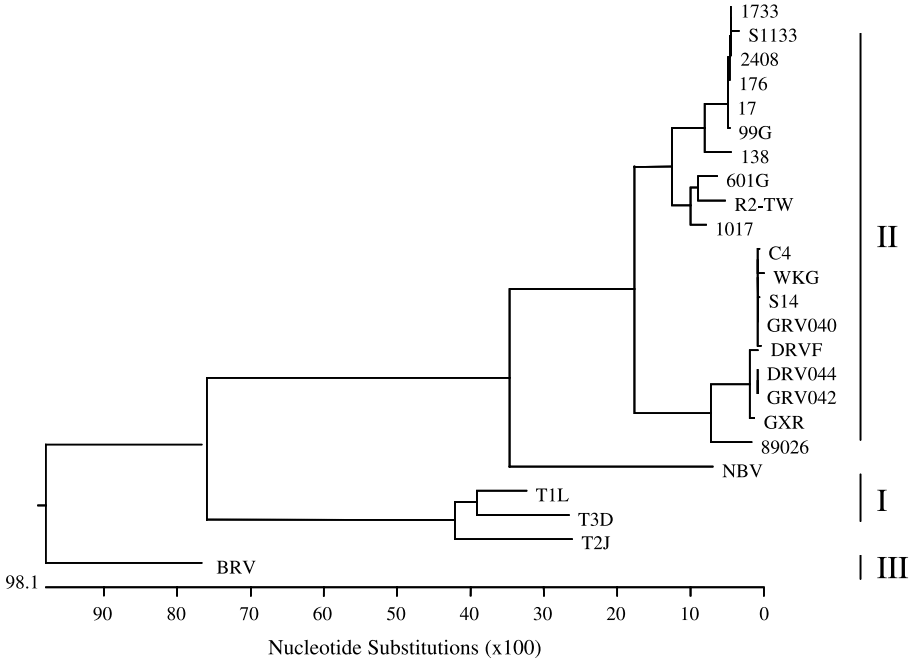

Fig. 4. Phylogenetic trees based on 597-bp nucleotide sequences (54 to 652) of the σA-encoding gene of reoviruses using the clustal program of the DNASTAR software package. The lengths of the horizontal lines are proportional to the minimum numbers of the nucleotide differences. The designations of the three species (*I–III*) are indicated. Sequences analyzed included the isolates from this study (Table 1), NBV, BRV, and MRV1-3 (T1L, T2J, and T3D)

of amino acid sequences of σA between DRV and ARV or GRV and ARV led us to group GRV and DRV closer to ARV than to NBV.

Discussion

Since the first reported case of duck reovirus in South Africa in 1950 [13], many isolates of duck and goose reovirus have been made from diseased as well as healthy-looking aquatic bird species during routine screening. To date, most detection of reovirus in infected ducks and geese has been carried out using classical methods, including propagation of the virus in a susceptible cell line, observations of virus particles by using electron microscopy, SDS-PAGE, immunofluorescence assay, and immunodiffusion gel assay. Most of these techniques are time-consuming or require special equipment, and may not be very sensitive. Despite several published methods based on nucleic acid RT-PCR detection for avian reovirus, the primers they used could detect only one individual species, the chicken reovirus (named avian reovirus) [2, 15, 18, 20]. Thus, a more universal set of primers is needed to ensure that a wider spectrum of various species (ARV, DRV, and GRV) could be detected in infected cell culture or carrier birds.

With this in mind, the development of an RT-PCR assay that could detect a wider range of bird species reovirus strains was undertaken. The assay required the design of a pair of novel degenerate primers to accommodate the degeneracy exhibited by most of the ARV and DRV sequences. Degenerate primers have been used successfully in RT-PCR detection of various human viruses [17, 34]. The approach of using the σA-encoding gene as target for RT-PCR resulted in reovirus amplification of cDNA from chickens, ducks, and geese robustly enough to detect a variety of geographically distinct strains isolated over the past 30 years. In the RT-PCR reactions that were carried out there was only one main product of ∼600 bp. No products were obtained when nucleic acids other than the genomic dsRNA of the specified reovirus mentioned above were used as RT-PCR template.

When the RT-PCR method developed above was tested for its sensitivity using DRV S14, as little as 0.01 PFU was sufficient to be detected.

The validity of the RT-PCR method for detecting clinical samples was also tested. Results indicated that the 600-bp specified product could be amplified from 85% (71/83) of DIF-positive liver samples. This RT-PCR reovirus assay detection from cell culture is more sensitive (about 15% higher) than amplifying directly from clinical specimens; we suspect that virus concentration in liver specimens is sometimes below the threshold for RT-PCR detection or that PCR inhibitors in clinical specimen hamper virus detection. However, in order to circumvent disease outbreaks caused by reovirus, the ability to diagnose viral infections early is of great importance to the poultry industry. Especially, virus propagation in cell culture costs more than 7 days and needs SPF embryo eggs; thus, the detection of RNA from clinical samples by RT-PCR is still the first choice in early diagnosis and would be of great importance to the poultry industry in order to circumvent outbreaks in the near future. This σA-encoding gene RT-PCR significantly improves the performance of currently available reovirus diagnostic

strategies. In addition, this method could be very useful in contributing to the creation of a databank of circulating reovirus sequences.

σA proteins, which possess dsRNA-binding activities [23, 35], revealed presumably functional constraints against amino acid changes. The homologies at the nucleotide and amino acid level showed that these viruses have been adapted to their hosts for a long time. The sequence region from amino acid residue 136 to 212 was highly conserved among ARV, DRV, and GRV, suggesting that this region is well adapted to different host species and might be function related.

The tree topologies based on 597-bp (54–652) sequences compared to that of full-length σA-encoding genes of ARV and DRV from the Genbank database were identical (data not shown). Therefore, the 597-bp sequence is a reliable region for phylogenetic analysis, and results will extend current knowledge about bird reovirus evolution and diversity. In paired identity analysis, there was over 98.4–100% nucleotide sequence identity between Chinese DRV and GRV isolates, suggesting that they should be members of the same species of virus and descend from a common ancestor. The 12% diversity of the σA-encoding gene between Chinese DRV and French DRV-89026 suggested that DRVs are evolving separately on different continents and represent distinct genogroups. The σA-encoding gene segment exhibited 74–78% nucleotide and 87–91% amino acids sequence identity, respectively, between DRV/GRV and ARV. By contrast, sequence comparisons of the DRV σA protein with MRV (T1L, T2J, and T3D), NBV, and BRV homologues revealed extreme sequence divergence, with 28–60% amino acid identities, which are consistent with previous ARV investigations [5], and support the classification of DRV/GRV in subgroup II of the genus *Orthoreovirus*, but distinct and separate from ARV and NBV. Phylogenetic distance seems related to the type of bird studied: duck and goose are from *Anseriforme*, which represent "primitive" birds, whereas chickens of the *Galliformes* represent more "advanced" birds.

Inter-genotype and multiple-gene reassortment have occurred in nature within ARV and mammalian reoviruses [3, 19], but the hypothesis of genetic reassortment between DRV/GRV and ARV genome segment needs more sequence data support. It is still unclear whether the duck/goose and chicken reoviruses derived from a common ancestor or not. Therefore, studies of more DRV and GRV genome segment sequences from field-isolate strains will extend our knowledge of phylogenetic relationships between DRV/GRV and ARV and other members of the family *Reoviridae*.

Acknowledgements

This work was supported by a grant from the National Basic Research Program (2005CB522905), China.

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