

Isolation and molecular characterization of Brazilian turkey reovirus from immunosuppressed young poult

Ana Carolina G. Rosa · Helena Lage Ferreira ·
Deriane Elias Gomes · Cilene Vidovix Táparo ·
Tereza Cristina Cardoso

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Abstract In this study, we investigated turkey reovirus (TReoV) in tissue samples from young birds, aged 15 days. RT-PCR for TReoV detected 3.3 % positive samples and TReoV was successfully isolated in Vero cells. Histological analysis of positive bursa of Fabricius (BF) revealed atrophied follicles and lymphocyte depletion. The number of CD8+, CD4+ and IgM+ cells was lower in infected BF. Phylogenetic analysis based on S3 gene showed that the Brazilian TReoV isolates clustered in a single group with 98–100 % similarity to TReoV strains circulating in the United States. This is the first indication that TReoV infection may be a contributing factor to immunosuppression in young birds.

The Brazilian turkey industry ranks second in the world. However, enteritis in young flocks continues to result in significant economic losses each year. Poult enteritis and mortality syndrome (PEMS), resulting from a coronavirus, was reported in 2006 [25]. Additionally, poult enteritis complex (PEC) has been associated with enteritis and growth rate reductions in commercial turkeys worldwide

[4–6, 13–15, 19–21, 23, 24]. To date, turkey coronavirus (TCoV), turkey astroviruses 1 and 2 (TAsV-1 and TAsV-2), and turkey rotavirus (TRoV) have been described to be circulating among poult breeders in Brazil [3, 7, 9, 18]. In contrast, turkey reovirus (TReoV) had never been detected so far.

Avian orthoreoviruses (ARVs) cause economically important diseases in poultry. They belong to the genus *Orthoreovirus*, subfamily *Spinareovirinae*, family *Reoviridae* [12]. ARVs have a segmented double-stranded RNA (dsRNA) genome comprised of three large (L1–L3), three medium (M1–M3), and four small (S1–S4) genomic segments [10]. In turkeys, a group of TReoVs has been described that are genetically distinct, based on sequence analysis of their S3 genomic segments, from the other members of the genus *Orthoreovirus* [10]. These TReoVs were originally isolated from commercial turkey flocks, especially those in the southeastern United States, affected by PEC [8, 13–15, 19–21, 23, 24]. TReoVs can induce enteric disease and immunosuppression in turkeys; their pathogenicity is restricted to poults, however [19]. The aim of the present study was to isolate and molecularly characterize the first documented TReoV affecting immunosuppressed young poults in Brazil.

Samples of young poults (15 days old) from a farm with more than 20,000 birds, kept in five separate houses, were investigated in September of 2012. The principal claim was related to low growth performance and weight losses. Samples were taken from individual houses containing approximately 4,000 birds each, and 14 % of the turkeys showed signs of deep depression but not of enteritis. Fifty samples from the bursa of Fabricius (BF), thymus, and intestinal contents and tissues were collected and sent to the laboratory. The samples were divided evenly into two groups. In the first group, samples were fixed in 10 %

A. C. G. Rosa · D. E. Gomes · C. V. Táparo · T. C. Cardoso
Laboratório de Virologia, Faculdade de Medicina Veterinária,
University of São Paulo State, Rua Clóvis Pestana, 793,
Araçatuba, SP CEP 16050-680, Brazil

H. L. Ferreira
Departamento de Medicina Veterinária, FZEA-USP, Av. Duque
de Caxias Norte, 225, Pirassununga, SP CEP 13635-900, Brazil

T. C. Cardoso (✉)
Departamento de Apoio, Produção e Saúde Animal, Curso de
Medicina Veterinária, Rua Clóvis Pestana, 793, Araçatuba,
SP CEP 16050-680, Brazil
e-mail: tcardoso@fmva.unesp.br

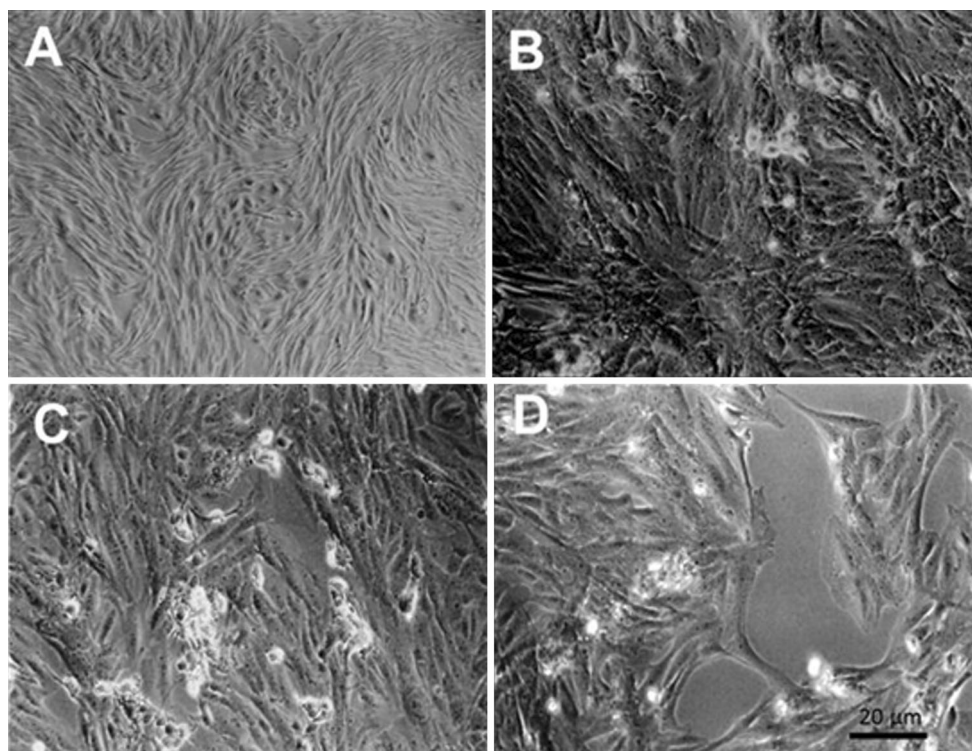


Fig. 1 Evaluation of TReoV isolated from bursa of Fabricius (BF). **A** Vero cells infected with a BF suspension prepared from healthy poults (control group). **B-D** Cytopathic effects were observed as

rounded adherent cells (**B**), fusion (**C**), and the generation of giant multinucleated and enlarged cells (**D**). Magnification, 40 \times ; bar, 20 μ m

neutral buffered formalin for histology analysis. In the second, samples were frozen for virologic procedures.

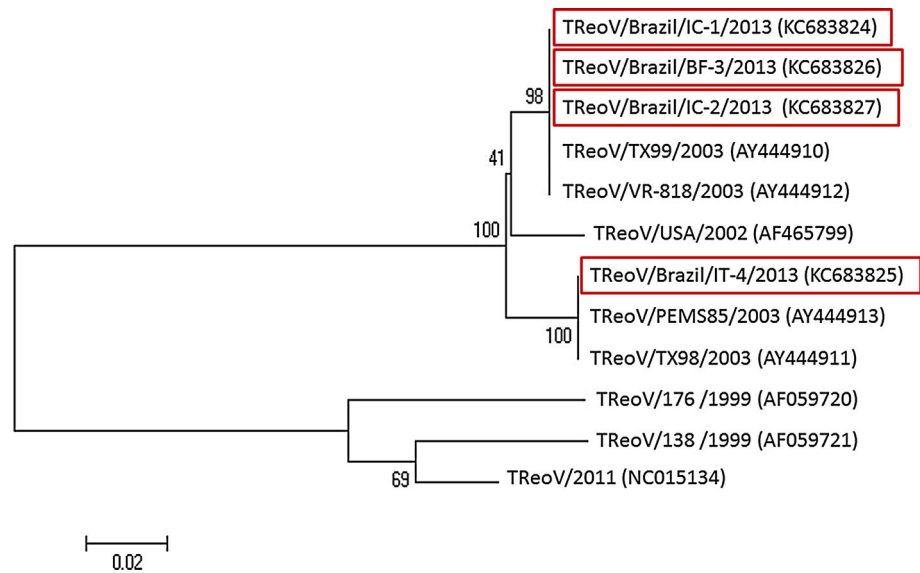
The latter samples were homogenized with a twofold volume of MEM (Sigma-Aldrich[®], St. Louis, MO, USA), clarified by centrifugation (2,500 \times g for 20 min), and filtered twice using 0.45- and 0.22- μ m syringe filters [9]. The suspensions were propagated in Vero cells for no more than four passages. When >80 % of the cells exhibited a cytopathic effect (CPE), the remaining adherent cells were scraped, and all cellular material was pelleted and frozen at -80°C for RNA extraction. All samples were tested by RT-PCR as described in a previous study [21] for the presence of TCoV, TAsV-1 and 2 and TRotV to exclude co-infections. TReoV was isolated only from BF suspensions of affected poults. Three consecutive blind passages in semi-confluent Vero cell monolayers resulted in rounded floating cells, followed by the generation of some giant multinucleated cells via fusion (Fig. 1).

To confirm TReoV infection, viral RNA was extracted from each sample (the BF, thymus, and intestinal contents and tissues) and also from the cell culture isolates using a Pure Link Viral RNA/DNA Kit (Invitrogen[®], Carlsbad, CA, USA) according to manufacturer's instructions. RT-PCR was performed as described previously [9, 11]. A portion of the δ 2 S3 gene was amplified using 10 pmol of

the oligonucleotide primers Reo S3F (5'-ATGGAGGTACGTGTGCCAAACT-3') and Reo S3R (5'-CCAACCACACTCCACAACAG-3') [8, 16, 22]. The expected products (1.1 kb) were analyzed by gel electrophoresis (1.5 % agarose gels with ethidium bromide) and UV illumination. TReoV infection was confirmed by amplifying the S3 gene using RT-PCR from Vero cells and BF tissue (data not shown). Although no virus was isolated from the intestinal contents and tissues, three samples were detected by RT-PCR.

The PCR products were purified using a commercial purification kit according to the manufacturer's instructions and sequenced directly using an ABI 3730xl DNA analyzer. Nucleotide sequences from this study have been deposited in GenBank under the following accession numbers: KC683824 and KC683827 (TReoV detected in intestinal contents), KC683825 (TReoV detected in intestinal tissues), and KC683826 (TReoV detected and isolated from the BF and Vero cells). Sequences for the δ 2 protein (S3) mRNA of AY444910 (TReoV TX99), AY444911 (TReoV TX98); AY444912 (TReoV ATCC VR-818), and AY44913 (TReoV PEMS 85) were obtained from GenBank for the phylogenetic analysis. Sequences were assembled using SeqMan Pro, and genome annotation was done using SeqBuilder (DNASTAR, Inc., v. 8.0.2).

Fig. 2 Phylogenetic relationship of avian and turkey reoviruses based on the $\delta 2$ amino acid sequences detected in commercial poult with immunosuppression. The sequences names are expressed as GenBank accession numbers; sequences starting with KC are from the present study. The Brazilian strains are marked with red boxes. The tree was constructed using the neighbor-joining method with Kimura's two-parameter correction, and bootstrap values are shown



Nucleotide and deduced amino acid sequences were aligned using Clustal-W in MegAlign (DNASTAR, Inc.). A phylogenetic tree was constructed based on amino acid alignments using BLAST analysis (www.blast.ncbi.nlm.nih.gov/Blast.cgi). The positive amplified S3 segments obtained from the BF suspension were genetically identical to the TReoV isolated from the same biological source. A phylogenetic tree showed that the nucleotide sequence from the present analysis had 98 % similarity to TReoV TX99 and ATCC/VR-818, and 100 % to TReoV PEMS 85 and TX98 (Fig. 2).

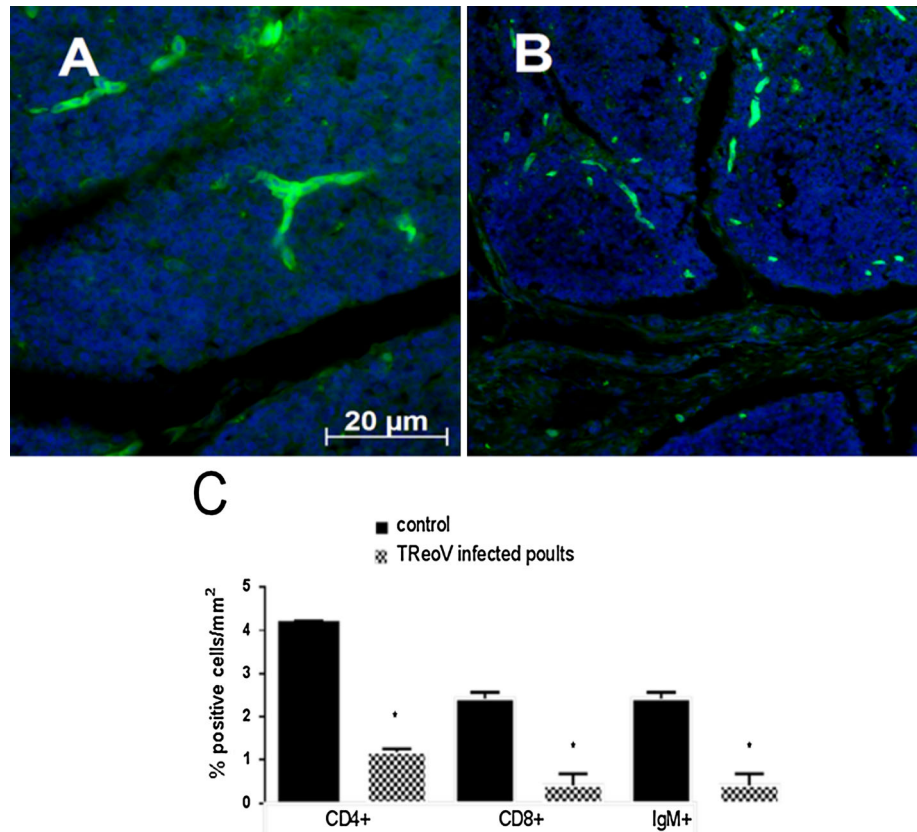
Tissues were fixed in 10 % neutral buffered formalin and embedded in paraffin [2, 9]. Sections were cut into 4- to 5- μ m thin sections and stained with standard hematoxylin and eosin (HE). In all cases, the BF was subjected to histological examination and scored based on lymphoid necrosis, lymphoid depletion, multifocal bursa hemorrhage, and follicle atrophy [19]. Damage intensity was scored semi-quantitatively using a four-point scale. A score of 1 represented mild damage; a score of 2, moderate damage; a score of 3, intense damage; and a score of 4 represented very intense damage.

Unstained sections (field samples and experimentally infected cells) were used for histology evaluation and IFA. The IFA was conducted on BF sections in order to analyze their lymphocyte subpopulations (CD4+, CD8+, and IgM+) as described previously [2]. Instead of using frozen sections, the IFA was performed with formalin-fixed paraffin-embedded BF sections collected from field samples with BF sections from healthy poult as a control. Heat-induced epitope retrieval was conducted first using citrate buffer (pH 6.1) for 15 min at 700 W as a pre-treatment for reactivating viral antigens, which are normally damaged by formaldehyde fixation [2, 9]. Sections were then

deparaffinized and rehydrated by washing with PBS containing 0.1 % Tween 80, and the slides were then boiled in water. Monoclonal antibodies against CD4+ and CD8+ (100 μ L/slide; mouse anti-chicken CD4+ and CD8+ FITC, MCA2164F and MCA2166F, AbD serotec, Bio-Rad Company) and IgM+ (100 μ L/slide anti-chicken IgM, Sigma-Aldrich) was applied overnight in the dark at room temperature in a 1:100 dilution in PBS with 5 % bovine serum albumin (BSA). The samples were washed as described previously [2], and rabbit anti-mouse conjugated to fluorescein (1:200; Sigma-Aldrich) was added to IgM+ sections. The samples were kept in the dark for 30 min at room temperature. The images were collected under an AxioImager® A.1 light and an ultraviolet (UV) microscope connected to an AxioCam®MRc (Carl Zeiss, Oberkochen, Germany). The images were processed using AxioVision® 4.8 software (Carl Zeiss) for each antigen, and values were determined by automatic counting of positive cells/mm². For CD4+, CD8+ and IgM+ cells, significant differences between groups were determined by one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant. Data are expressed as mean \pm standard deviation (SD). All statistical analyses were performed using Prism software (GraphPad®, CA, USA). Major histological damage was described as severe multifocal lymphoid follicle atrophy (data not shown) and was directly associated with a low number of CD4+, CD8+ and IgM+ lymphocyte cells ($p < 0.05$) (Fig. 3A, B and C).

The roles of different viruses in enteric disease have not been well defined, and in commercial flocks, it is not uncommon to find clinically normal birds infected with enteric viruses [5]. The present study describes, for the first time, immunosuppressed young poult (aged less than

Fig. 3 Quantification of CD4+, CD8+ and IgM+ cells using immunofluorescence assay. **A** CD4+ lymphocytes. **B** CD8+ lymphocytes (40×). **C** Quantification of lymphocyte subpopulations performed by AxionVision 4.8 software grid counts of positive cells in control and infected poult. Means \pm standard deviations with a * superscript differ significantly ($P < 0.05$)



15 days) that were affected by only TReoV. In spite of this finding, TAsV-1 and TCoV have also been described in commercial flocks in Brazil. The clinical sign most commonly associated with PEMS worldwide is enteritis [6, 13–15, 19–21, 23–25]. In a recent study, PEMS, TAsV-1 and TCoV were the viruses most frequently associated with enteric disorders that were detected in commercial Brazilian flocks [18].

The BF tissue suspensions were the only samples from which virus could be isolated. In order to promote TReoV isolation, other studies have used many cell types, such as primary chicken embryo fibroblasts, chicken embryo livers, kidney cells, and hepatocellular carcinoma cells, and Marek's disease turkey cells and lymphoblastoid cells [17, 22]. In all cell types, the cytopathic effects included detached enlarged cells, syncytia, cell death, and cell sloughing, which are similar to those described here. In recent studies, low passages of TReoV strains in Vero cells have been used to avoid genetic changes [19]. In this study, TReoV was passaged three times in Vero cells and then isolated with no genetic changes.

One effect of reovirus infection is immunosuppression, which predisposes the host to infection with other pathogens. This could account for the diversity of syndromes associated with reoviruses [11]. The bursal lymphoid depletion caused by TReoV isolates may induce transient and possibly

permanent immunosuppression in turkeys [19]. This was confirmed here by analyzing BF histological sections from field cases for the presence of CD4+, CD8+, and IgM+ lymphocytes. According to previous studies, severe BF lesions in the cortical and medullary areas are associated with positive lymphocytes [19]. Although several studies also describe intestinal lesions associated with both natural and experimental TReoV infection [13–15, 19–21, 23, 24], in this study, this kind of tissue damage was not documented.

A previous study demonstrated that programmed cell death, also known as apoptosis, could be the mechanism by which TReoV triggers disease in the host [1, 19]. This biological mechanism could explain, for instance, the lymphocyte depletion observed here, although lymphocytes are not considered a target for reovirus replication [11]. Importantly, the death of lymphocytes before two weeks of age can result in permanent immunosuppression [11]. The lack of inflammation in the BF and of histological changes in the thymus suggests that the infection observed in this study was in its initial phase. However, the observation of severe BF lesions and isolation of TReoV from these samples, along with the confirmation of TReoV in intestinal tissues and contents via PCR, means that these cases can be considered non-classical enteritis [1].

The $\delta 2$ protein sequences encoded by the S3 gene segments that were sequenced demonstrated that Brazilian

isolates clustered and were 98–100 % similar to USA isolates. Phylogenetic analysis demonstrated that these TReoV isolates separated from subgroups 1 and 3 orthoreoviruses isolates and clustered within the subgroup 2 clade [8, 16]. The results of this study indicate for the first time that this strain of TReoV shares genomic sequences and biological features with those described in flocks suffering from PEMS worldwide. Further studies are still necessary for investigating the pathogenicity of South American isolates, however.

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