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MICROBIOLOGY

# Constant testing for *Pestivirus* in cell lines reveals different routes of contamination

TATIANA F.P. DE OLIVEIRA, ANTÔNIO A.F. JÚNIOR, ANAPOLINO M. DE OLIVEIRA, MARCELO F. CAMARGOS & MARCOS B. HEINEMANN

**Abstract:** Pestivirus can contaminate cell cultures and sera and cause serious problems that evolve the integrity of studies, confidence in diagnostic results, and safety of human and animal vaccines. Contaminations by Pestivirus and other viruses may occur at any time and regular assays of monitoring in cell cultures and your supplies are necessary. This study aimed to analyze the phylogeny of Pestivirus detected from cell cultures, calf serum, and standard strains of three laboratories in Brazil that carry out frequent tests for the monitoring of cellular contaminations. These samples were submitted to phylogenetic analysis to understand the genetic relationship between contaminants occurring in these facilities. As result, the Pestivirus found in samples were Bovine viral diarrhea virus (BVDV-1 and BVDV-2), Hobi-like viruses (often named BVDV-3), and Classical swine fever virus (CSFV), and the phylogenetic analysis help us to infer at three possible routes of contamination in this work.

**Key words:** Pestivirus, phylogeny, cell line, contamination.

## INTRODUCTION

The genus Pestivirus, family Flaviviridae, is a group of single-stranded positive-sense RNA viruses that consists of four recognized species: Bovine viral diarrhea virus 1 (BVDV-1), Bovine viral diarrhea virus 2 (BVDV-2), Border disease virus (BDV) and Classical swine fever virus (CSFV) (Tautz et al. 2015, ICTV 2015, Smith et al. 2017). There are other putative species in this genus, like HoBi-like viruses (often named BVDV-3), initially detected in fetal calf serum but later associated with clinical disease in cattle (Bauermann & Ridpath 2015).

BVDV-1, BVDV-2, and Hobi-like viruses infect cattle causing acute, asymptomatic, or persistent infection. If symptomatic, the clinical signs in infected animals are fever, respiratory signs, reproductive disorders, and mild diarrhea (Weber et al. 2014). CFSV infects pigs and is listed by the World Organization of Animal Health as a notifiable disease that can impair the international commerce of animal products (Beer et al. 2015). Risk factors in pestivirus transmission include reproduction management, biosecurity measures, animal introduction, and herd size (Moennig et al. 2015).

Pestiviruses are also known as a contaminant of cell cultures (Pinheiro de Oliveira et al. 2013). Sera from large animals is the main component of nutrient cell media for cell culture and may be one important risk factor in contamination (Uryvaev et al. 2012). The presence of these viruses can lead to erroneous conclusions in studies of the mechanisms of virus-cell interaction or cause problems in vaccine production for humans or animals (Studer et al. 2002, Pastoret 2010).

BVDV is presented by two biotypes in nature, cytopathic and non-cytopathic strains

(Tautz et al. 2015). If cell contamination occurs by cytopathic strains, the culture will be discarded due to cell death. The major problem occurs with non-cytopathic strains that will cause persistent infection without apparent morphological disorders (Uryvaev et al. 2012).

This study aimed to analyze the phylogeny of Pestivirus detected from cell cultures, calf serum, and standard strains from three laboratories in Brazil that carry out frequent tests for the monitoring of cellular contaminations.

## MATERIALS AND METHODS

Samples from the three laboratories, one from a research and teaching institution (RT1) and two governmental laboratories (GO1 and GO2) involved in routine analysis and research, were routinely evaluated for contaminants as described by Pinheiro de Oliveira et al. (2013). Cell cultures and bovine fetal calf serum (FCS) positive for pestiviruses for one year in these laboratories were used in this work. The eight samples from RT1, were positive for pestivirus: three different MDBK (Madin-Darby bovine kidney epithelial cells) passages, one CC81 (kidney epithelia from cat) passage, one EBtr (embryonic bovine tracheal cells) passage, one FLK (follicular lymphoma), one RK13 (rabbit kidney) and one IBRS2 (pig kidney cells) passage. Pestiviruses were detected in two cell cultures (MDBK and RK13- rabbit kidney) in GO1 and two in FCS batches. In addition, 11 standard strains of Pestivirus used in GO1 were sequenced. One MDBK passage from GO2 was positive. In total, 11 cell cultures, 11 standard strains, and 02 FCS were studied in this work.

RNA was extracted using TRIzol® (Life Technologies, USA), according to the manufacturer's instructions. All nucleic acid extractions were evaluated using RT-qPCR for beta-actin and blank controls as described by Pinheiro de Oliveira et al. (2013). Protocol for Pestivirus 5' UTR detection was described by Ridpath & Bolin (1998). All amplicons were sequenced in ABI 3500 (Life Technologies, USA).

Phylogenetic trees were constructed using MEGA 6.06 (Tamura et al., 2013). Two trees were constructed using Neighbor-Joining and Maximum Likelihood models. The best model for nucleotide substitution proposed by the software was Kimura 2 parameters with gamma distribution.

Phylogenetic analyses are important to study the origin and evolution of pestiviruses (Mosena et al. 2022). We found similar results in both trees (Figure 1 and Figure 2). Sequences grouped with BVDV-1, BVDV-2, Hobi-like virus (BVDV-3), and CSFV. Clades separating Pestivirus species were supported by high bootstrap values. Two samples from FCS were used in GO1 grouped with Hobi-like virus, as well as one sample from RT1. HoBi-like viruses were found in other studies, sometimes ruining ruined much of the ongoing cell culture work because of contaminated FCS (Stahl et al. 2009).

## RESULTS AND DISCUSSION

Different passages of the same cell line in different laboratories were positive for pestivirus. Sequences from RK13 form GO1 and RT1 grouped with BVDV-1 strain HP-KY-RK13 [14]. These three sequences were identical to each other, indicating that this cell line may be persistently infected with BVDV. RTI1-IBRS-2D.p272 had 100% identity with HCV5UTRAN CSFV strain "IBRS2" in a high bootstrap clade, indicating another case of persistent infection by CSFV. GO1-SK6.p66 also seems to be another case of persistent infection, as it was very similar to standard and vaccine CSFV strains.

Different passages of the same standard BVDV strains were also sequenced. These


**Figure 1. The evolutionary history by the Neighbor-Joining method. The optimal tree with the sum of branch length = 1,24082590 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with 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 Kimura 2-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The analysis involved 62 nucleotide sequences. Sequences from Pestivirus detected in ☐ cell lines from GO2 ▲ cell lines from RT1 ● cell lines and CSF from GO1○ reference strains form GO1.**

passages were identical in the 5´ UTR region but also had a high similarity to RTI1-MDBK.p29 and RTI1-MDBK.p143.

Cell culture contamination may be prevented by constant testing of cell lines and reagents used. It is important to eliminate those lines that are infected to avoid errors in research and risks in the dissemination of viruses in biological products. For example, one hypothesis is that Hobi-like viruses originated in South America and were introduced to other continents through FCS and vaccines (Ståhl et al. 2007).

## CONCLUSION

The laboratories participating in this study constantly test reagents and cell lines for Pestivirus, porcine parvovirus, Mycoplasma sp., bovine leukemia virus, and porcine circovirus 2. All cells used in GO2 are provided by GO1. GO1 and RT1 exchange cell cultures and reagents constantly. Our phylogenetic analyses demonstrated that at least in two cases, cell contamination occurred due to the exchange of persistently infected cell lines as occurred with RK13 and IBRS2. These two cell lines were the target of other studies that demonstrated the presence of pestiviruses in multiple passages (Nam et al. 2015, Stadejek et al. 1996). Falcone et al. (2003) demonstrated that a non-cytopathic strain of BVDV-2 isolated from a batch of live infectious bovine rhinotracheitis vaccine was capable of developing severe signs of disease in calves inoculated intranasally.

Contamination by CSFV should be addressed carefully if these cell lines are manipulated or the biological products used are free of these

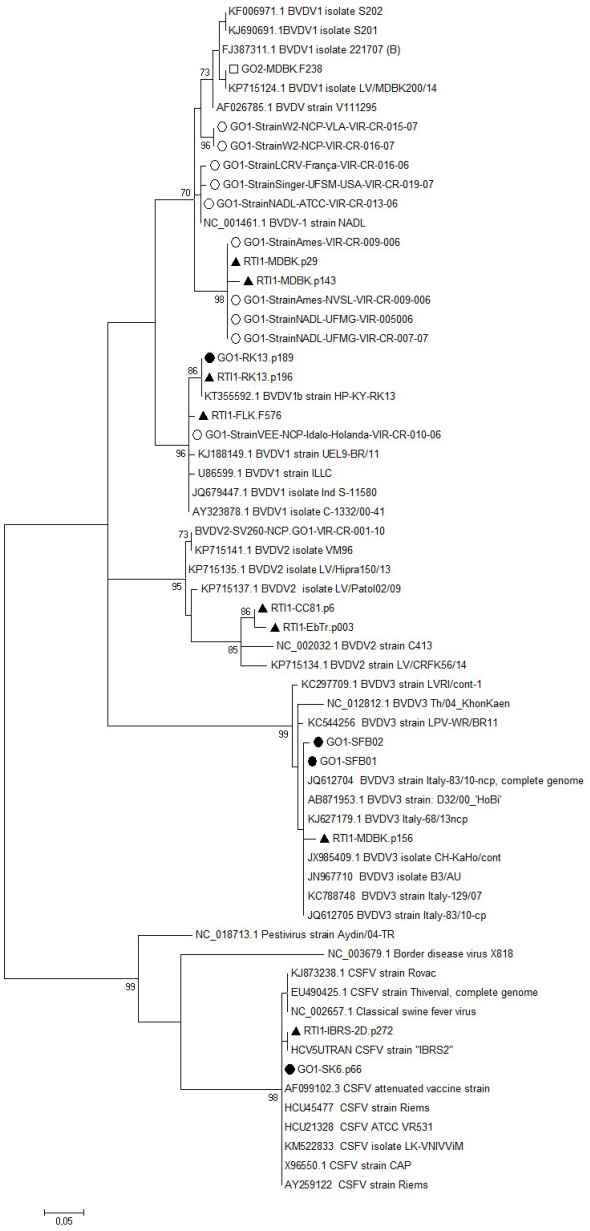


**Figure 2. The evolutionary history by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-887,5220) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0,8912)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 62 nucleotide sequences. Sequences from Pestivirus detected in ☐ cell lines from GO2 ▲ cell lines from RT1 ● cell lines and CSF from GO1○ reference strains from GO1.**

viruses like most regions important in the swine industry in Brazil.

Another possibility of contamination is the use of standard strains. Different passages of MDBK (RTI1-MDBK.p29 and RTI1-MDBK.p143) were contaminated by viruses very similar to those used in tests like virus neutralization. Even these strains may have been contaminated by each other, as they grouped with 100% similarity. Samples may be differently labeled in the laboratory and used in experiments with other names or can contaminate other studies inducing erroneous interpretations (Worobey et al. 2008).

We found two batches of CFS and one MDBK cell line contaminated by Hobi-like viruses. More than 30% of CFS batches from South America tested in Europe are contaminated by these Pestivirus species (Bauermann & Ridpath 2015). Hobi-like viruses are present in Brazilian cattle, so there is a high chance of contamination of cell lines by biological products produced from bovine. All CSF batches and cell lines should be constantly tested for Pestivirus using primers that may amplify RNA from different species and all strains circulating in the country.

BVDV-1 and BVDV-2 may also contaminate CSF (Uryvaev et al. 2012). GO2-MDBK.F238 grouped with sequences from BVDV-1 isolates from Brazil (Silveira et al. 2015). This contamination may be explained by the use of contaminated CSF.

We detected three possible routes of contamination in this work: cell lines exchange between laboratories, contaminated reagents, and contamination by reference strains. We used different primer sets to detect contamination. Real-time PCR did not detect all

strains (data not shown). Only the use of generic primers for Pestivirus was efficient to detect all contamination. Thus, constant testing and the correct PCR are extremely important to avoid persistent contamination of cell lines by this virus genus.

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**TATIANA F.P. DE OLIVEIRA**[1]
https://orcid.org/0000-0001-8805-9039

**ANTÔNIO A.F. JÚNIOR**[1,2]
https://orcid.org/0000-0002-2962-5616

**ANAPOLINO M. DE OLIVEIRA**[1]
https://orcid.org/0000-0001-3256-2258

**MARCELO F. CAMARGOS**[1]
https://orcid.org/0000-0001-8457-7875

**MARCOS B. HEINEMANN**[3]
https://orcid.org/0000-0001-6444-9788

[1]Laboratório Nacional Agropecuário de Minas Gerais, Laboratório de Biologia Molecular/Laboratório de Diagnóstico de Doenças Virais, Avenida Rômulo Joviano, s/n, 33600-000 Pedro Leopoldo, MG, Brazil

[2]Centro Universitário de Sete Lagoas, Av. Marechal Castelo Branco, 2765, Santo Antonio, 35701-242 Sete Lagoas, MG, Brazil

[3]Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Medicina Veterinária e Saúde Animal, Av. Prof. Dr. Orlando Marques de Paiva, 87, Butantã, 05508-270 São Paulo, SP, Brazil

Correspondence to: **Antônio Augusto Fonseca Júnior**
*E-mail: antonio.biomol@gmail.com*

## Authors contributions

Tatiana Flávia Pinheiro de Oliveira: RNA extraction, RT-PCR, sequecing. Antônio Augusto Fonseca Júnior: experiment design, sequecing, phylogenetic analysis, manuscript preparation. Anapolino Macedo de Oliveira: cell culture preparation. Marcelo Fernandes Camargos: cell culture preparation, manuscript preparation. Marcos Bryan Heinemann: experiment design, manuscript preparation.