SHORT COMMUNICATION

Genetic characterization of bovine herpesvirus 4 (BoHV-4) isolates from Argentine cattle suggests a complex evolutionary scenario

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Abstract

Bovine herpevsirus 4 (BoHV-4) is a gammaherpesvirus that has been associated with different clinical conditions in cattle. In Argentina, BoHV-4 was detected in diverse bovine samples. The aim of this study was to analyze the genetic relationship of 48 field BoHV-4 strains isolated from cattle in Argentina. According to thymidine kinase (*tk*) gene sequences, BoHV-4 isolates belong to genotypes 1, 2 and 3. Phylogenetic analyses confirmed the presence of the three previously described viral genotypes. However, some of the studied isolates presented conflicting phylogenetic signals between the studied markers. This suggests a complex evolutionary background, that is a history of recombination, incomplete lineage sorting (deep coalescence) or a combination of these, which requires further study. These potential events make difficult the diagnosis of BoHV-4 from clinical samples of cattle and may pose a significant problem for the control of the virus in the herds.

Keywords BoHV-4 · Argentinean strains · Phylogenetic analyses

Introduction

Bovine gammaherpesvirus 4 (BoHV-4) is a ubiquitous virus and the majority of BoHV-4 affected animals exhibit persistent and asymptomatic infection [1, 2], which highlights that through co-evolution with the host, BoHV-4, as other gammaherpesviruses, has developed mechanisms to control the immune response and to persist in the host, most of the time, in a symbiotic relationship. In Argentina, BoHV-4 was isolated from vaginal secretions of aborted cows [3], nasal swabs, brain tissue, oocytes, granulosa cells [4], semen from an artificial insemination center [5] and aborted bovine fetus [6]. The virus was also isolated from buffy coat fractions in association with bovine viral diarrhea virus (BVDV). Furthermore, in a previous work, we isolated BoHV-4 from one out of eleven peripheral blood leukocyte (PBL) samples from one herd [7]. Overall, this work demonstrated that BoHV-4 is present in the leukocyte fraction of dairy

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cattle and the viral strains present in this herd are genetically divergent. Only a few studies have documented the genomic analysis of potentially pathogenic field isolates of the virus [9]. The purpose of the present study was to perform a phylogenetic analysis of field BoHV-4 strains isolated from different bovine samples in Argentina.

Forty-eight BoHV-4 field strains were obtained from different samples from cattle belonging to 35 farms (Table 1). Samples included vaginal discharges, buffy coat, semen, lung, oocytes and lymph nodes, which were processed for virus isolation and inoculated in triplicate in 96-well plates and observed daily for the presence of cytopathic effect (CPE). Blind passages were performed every 48 h, and at the third blind passage, samples were routinely tested for the presence of bovine herpesvirus 1 (BoHV-1) and BVDV antigens by direct immunofluorescence (DIF) testing using labeled polyclonal antibodies (BVDV FITC Conjugate VMRD, N C-J-F-BVDV; IBR/BHV-1 FITC Conjugate VMRD, C-J-F-IBR]). Furthermore, we evaluated other pathogens associated with reproductive disorders in cattle and all samples were negative for BoHV-1 and 5. Some BoHV-4 adapted strains may grow easily in MDBK cells. However, most field strains do not replicate easily in cell cultures. Thus, samples were maintained in MDBK cells for up to ten blind passages. When CPE was observed and the presence of BoHV-4 was confirmed by nested PCR, the viral stock was amplified and stored at -80 °C. DNA from the forty-eight (48) BoHV-4 strains was purified from infected cells using the DNeasy Blood & Tissue Kit (Cat. 69504, Qiagen), according to the manufacturer's instructions. The presence of BoHV-4 DNA was evaluated by PCR assays targeting two ORFs of the viral genome: glycoprotein B (ORF8), as described by Wellenberg et al. [10] and *tk* gene (ORF 3), which was evaluated by a nested PCR modified by Verna et al. [8]. The primers used for the amplifications are described in (Table S1). For the phylogenetic analyses, two datasets were considered, the ORF3, encoding the tk gene and the ORF8 encoding the glycoprotein B. DNA from mock-infected MDBK cells was used as negative control and the specificity of each PCR assay for BoHV-4 was evaluated using DNA templates from a reference strain of BoHV-1 (Cooper strain) and a field BoHV-5 isolate (97/613).

Strains sequences were aligned with MAFFT using default op and ep, and iterative refinement and weighted sum-of-pairs and consistency scores obtained from local alignments [11]. The appropriate sequence evolution model for the phylogenetic analysis was identified using jModelTest 2 [12]. Phylogenetic trees were inferred with a maximum likelihood frame by means of RAxML-NG. Previously characterized strains sequences allowed the genotyping of the new strains incorporated to this study. The comparison of tree topology was performed using "cophyloplot" function available in the R package APE [13]. Maximum Likelihood phylogenetic trees were inferred with the FastTree 2 program [14]. For the bootstrapped analyses, 100 resampled datasets were generated with the Seqboot component of the Phylip package [15], and subjected to FastTree 2 and the CompareToBootstrap.pl PERL script provided with the program. The herein reported nucleotide sequences have been assigned to GenBank accession numbers MK095351 to MK095406.

The first record of the presence of BoHV-4 in Argentina was the case 07_435 from an aborted cow. Other pathogenic organisms which could be responsible for this abortion, including BoHV-1, BVDV and bacterial pathogens, were not identified. Fig. S1 shows the geographical distribution of all BoHV-4 genotypes circulating in different provinces of Argentina. As observed in Fig. S1, distribution of genotype 1 and not classifiable genotypes overlaps with the geographical distribution of genotype 2 strains.

The phylogenetic tree from *tk* gene sequences indicated the presence of three distinct groups corresponding to the viral genotypes 1 to 3 described previously [8]. Most of the new sequences generated in this work could be grouped into one of these phylogenetic groups (Fig. 1). As described before [8], the sequences from strains 08_415 and 08_404, could not be assigned to any of the three genotypes in the *tk* phylogenetic tree. Nevertheless, these strains grouped with genotype 3 sequences in the gB tree. Therefore, these two strains cannot be assigned to a single genotype. Likewise, sequences from strains semen_M5, semen_M1 and FMV9 [16] clustered near the genotype 1 sequences in the *tk* phylogenetic tree but were grouped within genotype 3 in the gB genealogy. In addition, strains 13_mcv5 and 10_441BC, could not be assigned to any genotype due to discordance between the *tk* and gB trees. The previously described American reference strain (DN599), which was assigned to genotype 2 according to the *tk* tree topology, clustered differently in the gB phylogeny.

Phylogenetic analysis of 48 BoHV-4 strains isolated from different samples revealed the association of distinct and novel genotypes of BoHV-4 with diverse clinical conditions in cattle. Our evolutionary analyses corroborated the presence of three circulating viral genotypes [8]. Interestingly, it can also be inferred that the genome of some strains presents divergence regarding their membership to the currently described genotypes. For example, the *tk* sequence from the strain 10_441BC_16 groups in genotype 3 whereas the corresponding gB sequence cluster with genotype 2 reference sequences (Fig. 1). Other cases are even more complex, such as reference strain DN599 that clusters in genotype 2 according to the *tk* sequence. However, grouping with any of the previously described genotypes in the gB phylogeny could not be established for this strain. This intricate phylogenetic scenario suggests that BoHV4 diversity responds to phenomena other than simple nucleotide substitutions and *indels*, such as deep coalescence, incomplete lineage sorting

Table 1 Field Argentinean BoHV-4 isolates analyzed in this study

Isolates (ID)	Location	Province of origin	Clinical condition	Clinical sample	Genotype	GenBank accession
07_435_11	Vedia	Buenos Aires	Abortion	Vaginal discharges	3	KU180421.1 (tK)/ KU180390.1 (gB)
07_568_11	Trenque Lauquen	Buenos Aires	Abortion	Vaginal discharges	2	KU180422.1 (tK)/ KU180391.1 (gB)
08-209_11	Carmen de Areco	Buenos Aires	Abortion	Vaginal discharges	2	JQ838049.1 (tK)/ KP209018.1 (gB)
08-263_11	Gral. Pueyrredon	Buenos Aires	Abortion	Vaginal discharges	2	This study
08-330_16	Pehuajo	Buenos Aires	Abortion	Vaginal discharges	2	This study
08-362_11	Gral Belgrano	Buenos Aires	Abortion	Vaginal discharges	2	JQ838051.1 (tK)/ KP209020.1 (gB)
08-404_11	Balcarce	Buenos Aires	Abortion	Vaginal discharges	Not classifiable	JQ838052.1 (tK)/ KP209021.1 (gB)
08-415_11	Bahia Blanca	Buenos Aires	Abortion	Vaginal discharges	Not classifiable	JQ838053.1 (tK)/
08-433_11	Pehuajo	Buenos Aires	Abortion	Vaginal discharges	2	JQ838054.1 (tK) KP209022.1 (gB)
08-476_11	Pehuajo	Buenos Aires	Abortion	Vaginal discharges	2	JQ838055.1 (tk)/ KP209023.1 (gB)
semen_M5	CABA	Buenos Aires	Unkown	Semen	Not classifiable	This study
semen_M1_16	CABA	Buenos Aires	Unkown	Semen	Not classifiable	This study
09-227_11	Gral Alvear	Buenos Aires	Abortion	Vaginal discharges	3	KU180423.1 (tK)/ KU180392.1 (gB)
09-465_11	Gral. Guido	Buenos Aires	Abortion	Vaginal discharges	2	JQ838057.1 (tK)/ KP209025.1 (gB)
09-467_11	9 de Julio	Buenos Aires	Abortion	Vaginal discharges	2	This study
09-508_11	Villa Maria	Cordoba	Abortion	Vaginal discharges	1	KU180418.1(tK)/ KU180394.1(gB)
09-759_11	S M del Monte	Buenos Aires	Abortion	Vaginal discharges	2	KU180419.1 (tK)/ KP209027.1 (gB)
10-154_11	Venado Tuerto	Santa Fe	Abortion	Vaginal discharges	2	KU180416.1 (tK) KP209028.1 (gB)
10-315_MCV8	25 de Mayo	Buenos Aires	Abortion	Vaginal discharges	2	This study
10-315_MCV9	25 de Mayo	Buenos Aires	Abortion	Vaginal discharges	2	This study
10-315_MCV10	25 de Mayo	Buenos Aires	Abortion	Vaginal discharges	2	This study
10–322	CABA	Buenos Aires	Abortion	Vaginal discharges	3	This study
10-441BC	Mar chiquita	Buenos Aires	Unknown	PBL	Not classifiable	This study
10-347_BC	Achira	Cordoba	Unknown	PBL	2	This study
10-347_mcv5	Achira	Cordoba	Abortion	Vaginal discharges	2	This study
10–514	9 de Julio	Buenos Aires	Abortion	Vaginal discharges	3	This study
11-859_mcv13	9 de Julio	Buenos Aires	Abortion	Vaginal discharges	3	This study
11-862_8363_16	Tartagal	Salta	Abortion	Vaginal discharges	3	This study
11-862_23770_16	Tartagal	Salta	Abortion	Vaginal discharges	3	This study
11-862_4573	Tartagal	Salta	Abortion	Vaginal discharges	3	This study
11-862_32487_16	Tartagal	Salta	Abortion	Vaginal discharges	3	This study
11-862_c416_16	Tartagal	Salta	Abortion	Vaginal discharges	3	This study
11-862A654	Tartagal	Salta	Abortion	Vaginal discharges	3	This study
Granulosa_14	Balcarce	Buenos Aires	Unkown	Granulose cells	2	KP209014.1 (tK)/ KP209030.1 (gB)
12-365_14	Gral Villegas	Buenos Aires	Abortion	Vaginal discharges	2	KU180417.1 (tK)/ KU180395.1 (gB)

Table 1 (continued)

Isolates (ID)	Location	Province of origin	Clinical condition	Clinical sample	Genotype	GenBank accession
12-512_14	Azul	Buenos Aires	Abortion	Vaginal discharges	2	KU180420.1 (tK)/ KU180393.1 (gB)
13-13mcv5_16	Henderson	Buenos Aires	Abortion	Vaginal discharges	Not classifiable	This study
13-13mcvb4_16	Henderson	Buenos Aires	Abortion	Vaginal discharges	2	This study
13-294_mcv	Italo	Cordoba	Abortion	Vaginal discharges	3	This study
13-331_mcv261	Pehuajo	Buenos Aires	Abortion	Vaginal discharges	2	This study
13-350mcv_16	Pehuajo	Buenos Aires	Abortion	Vaginal discharges	2	This study
13-361mcv	Venado Tuerto	Santa Fe	Abortion	Vaginal discharges	2	This study
13-Ovocito-H3	Balcarce	Buenos Aires	Unkown	Ovocites	2	KP209031.1 (gB)
H140105_14	Rafaela	Santa Fe	Abortion	Vaginal discharges	2	This study
15-413mcv3_16	Azul	Buenos Aires	Abortion	Vaginal discharges	2	This study
15-431mcv_16	Pehuajo	Buenos Aires	Abortion	Vaginal discharges	2	This study
15-760_pulmon_16	Carmen de Areco	Buenos Aires	Respiratory	Lung	2	This study
BCS_16	Tandil	Buenos Aires	Unkown	PBL	3	This study
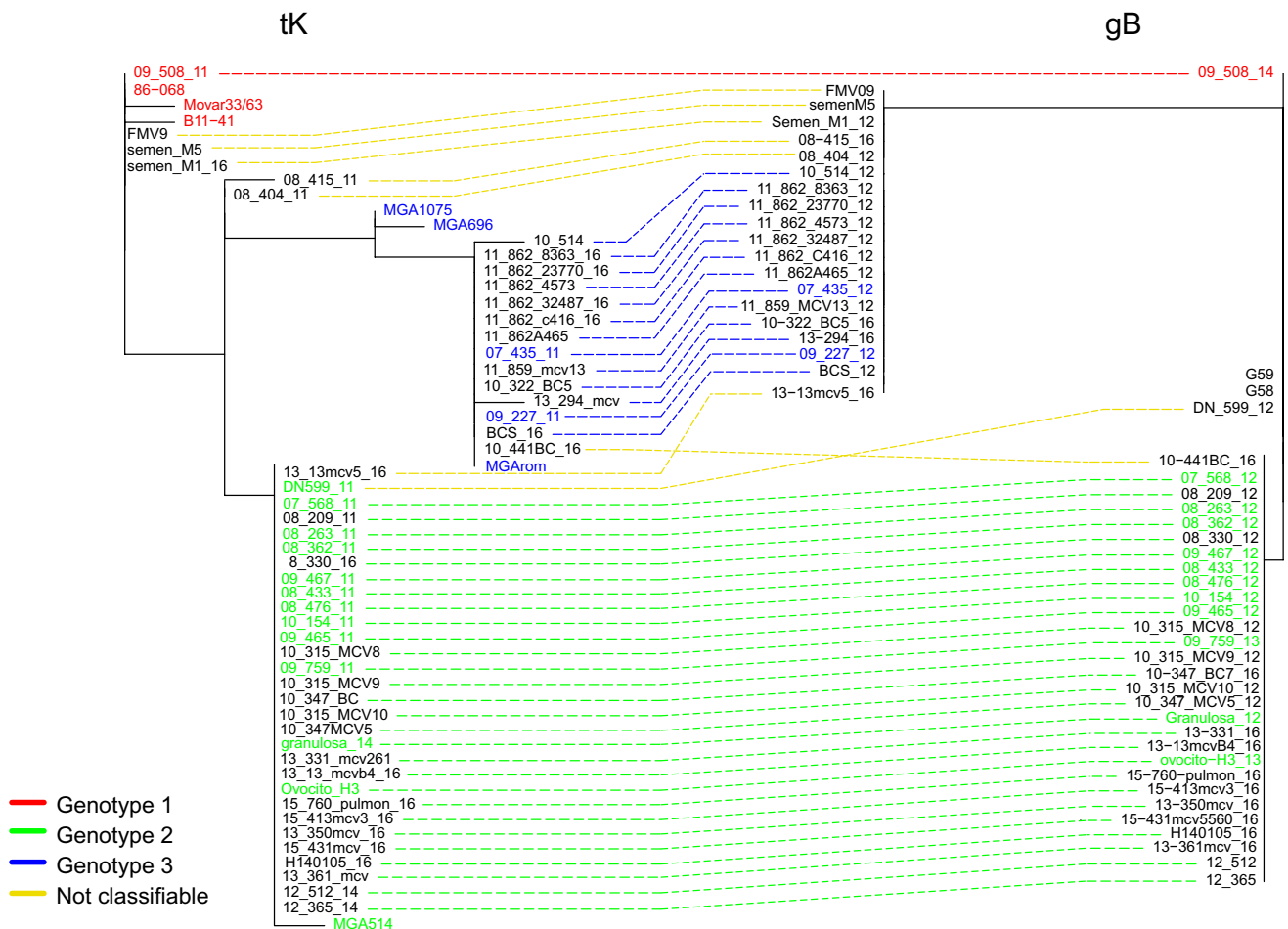
Fig. 1 Cophyloplot depicting gene trees obtained for the *tk* (left) and *gB* (right) sequences. Terminal colors indicate genotypes as coded in the inset legend. The strains whose names are colored in black and for which the cophyloplot links (CL) are colored in yellow correspond to strains that displayed discordant relationships between the *tk* and *gB* gene trees. In contrast, the CL corresponding to strains that displayed concordant genotype assignments are colored according to the assigned genotype. The tree roots were placed arbitrarily for an enhanced display

and/or homologous recombination. The present study might indicate that microevolutive changes due to intra-specific recombination have also occurred leading to the clear divergence between strains. The impact of the markedly complex genetic and evolutionary background of the virus in cattle production and health clearly deserves further investigation. The data presented here compiles the information of an important number of circulating field BoHV-4 strains which contributes to the characterization of the impact of this emerging virus in Argentina.

Compliance with ethical standards

Conflicts of interest None of the authors have any competing interests in the manuscript.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Research involving human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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