Efficient assembly of full-length infectious clone of Brazilian IBDV isolate by homologous recombination in yeast

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Abstract

The Infectious Bursal Disease Virus (IBDV) causes immunosuppression in young chickens. Advances in molecular virology and vaccines for IBDV have been achieved by viral reverse genetics (VRG). VRG for IBDV has undergone changes over time, however all strategies used to generate particles of IBDV involves multiple rounds of amplification and need of in vitro ligation and restriction sites. The aim of this research was to build the world’s first VRG for IBDV by yeast-based homologous recombination; a more efficient, robust and simple process than cloning by in vitro ligation. The wild type IBDV (Wt-IBDV-Br) was isolated in Brazil and had its genome cloned in pJG-CMV-HDR vector by yeast-based homologous recombination. The clones were transfected into chicken embryo fibroblasts and the recovered virus (IC-IBDV-Br) showed genetic stability and similar phenotype to Wt-IBDV-Br, which were observed by nucleotide sequence, focus size/morphology and replication kinetics, respectively. Thus, IBDV reverse genetics by yeast-based homologous recombination provides tools to IBDV understanding and vaccines/viral vectors development.

Key words: infectious bursal disease virus, reverse genetics, yeast-based homologous recombination.

Introduction

Infectious bursal disease virus (IBDV) is a pathogen causing immunosuppressive disease in young chickens that multiplies rapidly in B lymphocytes precursors in the bursa of Fabricius (BF) (Jackwood et al., 1982). The IBDV infection may lead to immunosuppression and increased susceptibility to other diseases, causing significant losses to the poultry industry (Balamurugan and Kataria, 2006). In Brazil, the disease was first described by Nakano et al. (1972) through the pathological diagnosis. In 1978 Saukas and collaborators reported the virus isolation (Saukas, 1978) and cases of mortality due to very virulent IBDV (vvIBDV) were observed in this country in 1997 (Di Fabio et al., 1999).

IBDV is a member of the family Birnaviridae, genus Avibirnavirus and contains a double-stranded RNA (dsRNA) genome consisting of two segments designated A and B (Müller et al., 1979). The larger segment, A (3.2 kb), contains two partially overlapping open reading frames.
(ORFs 1 and 2). ORF1 encodes a 17-kDa nonstructural protein (VP5) present in IBDV infected cells (Mundt et al., 1995). ORF2 encodes for a precursor polypeptide (110-kDa), which is processed to produce the mature structural proteins VP2, VP3 and VP4 (Azad et al., 1985; Hudson et al., 1986). The smaller segment, B (2.8 kb), encodes VP1, a 97-kDa multifunctional protein with polymerase and capping enzymatic activities (Pan et al., 2007; von Einem et al., 2004).

Reverse genetics has significantly increased the knowledge on several aspects relating to the IBDV. The first reverse genetics system to IBDV was created in 1996 with the generation of IBDV by cellular transfection of RNAs produced by in vitro transcription (Mundt and Vakharia, 1996). Advances came by the use of cloned viral cDNA transfection (Boot et al., 1999; Lim et al., 1999), which has been shown to be a better method than RNA transfection to rescue recombinant IBDV (Boot et al., 2001). The clone viral cDNA transfection method has passed for additional modifications (Ben Abdeljelil et al., 2008; Qi et al., 2007) and now has been used to study biological functions and vaccines development (Gao et al., 2011). Nowadays, the reverse genetics technology is the main tool for construction of IBDV recombinant attenuated vaccines, such as vector-based (Zanetti et al., 2012), chimeric vaccines (Gao et al., 2011) and virus-like particles (VLPs) production (Wang et al., 2012).

However, these different reported strategies of reverse genetics for IBDV involve multiple rounds of amplification, use of specific restriction sites and the need of in vitro ligation for assembly plasmids to generate IBDV particles. These strategies are tiresome, time consuming to perform and require many steps. However, in 1997, the observation that linear DNA fragments can stimulate recombination in Saccharomyces cerevisiae, led to a rapid development of methods for DNA manipulation in yeast (Oldenburg et al., 1997), such as yeast-based homologous recombination cloning technique. In this methodology, a DNA fragment containing homologous ends with the vector can be directly cloned using in vivo recombination in a linearized vector by an efficient, robust and simple process (Gibson, 2009; Shanks et al., 2009).

In the present study, we isolated IBDV from Brazilian commercial chicken (wild type IBDV-Br, Wt-IBDV-Br) with clinical signals suggestive of IBDV infection, amplified its genome and confirmed its identity by sequencing. After complete virus genome amplification, we built and characterized, in cell culture, the first reverse genetics system of IBDV made by homologous recombination in yeast. This new biotechnology tool can be used to study the IBDV molecular virology and new vaccines development.

### Material and Methods

#### Cell culture and IBDV Isolation

Chicken embryo fibroblasts (CEF) cells (Embrapa Swine and Poultry, Santa Catarina, Brazil) were grown in F10/M199 medium containing 10% fetal bovine serum (FBS) supplemented with 1% Penicillin and Streptomycin (LG Biotecnologia, Cotia, SP, Brazil), 0.025% Neomycin Sulfate (LG Biotecnologia, Cotia, SP, Brazil) and 0.1% Fungizone (Gibco, Langley, OK, EUA). Cells were grown at 37 °C in a humidified 5% CO2 incubator (conditions maintained in all work).

The Wt-IBDV-Br strain was isolated from commercial chickens with signs of weight loss, BF irregular size, apathy and poor appetite. Spleen and BF from five of these commercial chickens with 26-27 days old with clinical signals suggestive of IBDV infection were processed in duplicate (Romero, 1987; Reynolds, 1998). The spleen and BF were macerated and suspended in sterile Phosphate Buffered Saline (PBS) 1/10 (weight/volume) supplemented with antibiotics (Romero, 1987; Reynolds, 1998). The macerated tissue was centrifuged at 3000 g for 15 min and 0.2 mL of supernatant was inoculated (in triplicate) in nine day old embryonated chicken eggs (Embrapa Swine and Poultry, Specific Pathogenic Free, SPF). The inoculation was performed in the chorioallantoic membrane (CAM), 0.2 mL/egg. After inoculation, eggs were incubated at 37 °C and monitored for seven days by ovooscopy prior isolation steps. The negative control was performed with PBS inoculated in CAM of SPF eggs (Romero, 1987; Reynolds, 1998). The following steps for Wt-IBDV-Br isolation involved three successive inoculations in embryonated SPF eggs (0.2 mL/egg) using the chorioallantoic liquid as a virus source and then seven successive passages in CEF (1 mL/passage in CEF) (titers were not measured between passages). The virus recovered in the last cell culture step was identified by sequencing of VP2 region and used to construct the reverse genetics system of IBDV.

The sequencing was performed with viral RNA (vRNA) extracted from 150 μL of CEF cell culture supernatant infected with Wt-IBDV-Br by Viral RNA Isolation Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. vRNA was eluted from the column in a final volume of 50 μL (160 ng/μL) of RNase-free water and stored at -80 °C until required.

The reverse transcription (RT) to VP2 region was performed in total 20 μL reaction by mixing 2 μg of vRNA with 5 μM of VP2R primer (Table 1) and 200 U of Superscript III RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The cDNA was amplified with Phusion High-Fidelity DNA Polymerase 100 U (2 μL/μL) (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. PCR’s conditions were: 98°C for 30 s, followed by 32 cycles of 98 °C for 10 s, 54 °C for 30 s, 72 °C for
The primers used to amplification step (VP2-F and VP2-R) are showed in Table 1.

After analyze of nucleotide sequence (made as described below), the identity of the isolated was confirmed by comparison between the obtained sequence and the available GenBank sequences (National Center for Biotechnology Information, NCBI).

Sequence analysis
To confirm the sequence of Wt-IBDV-Br and validate plasmids constructs, nucleotide sequence was determined with the ABI BigDye terminator cycle sequencing on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). The sequence identity was confirmed by comparison amplified regions vs. NCBI sequences.

Amplification of the segment A and B
After vRNA extraction from cell supernatant (160 ng/μL), RT to A segment was performed in total 20 μL reaction with 5 μM of IBDV A-R recombination reverse primer (Table 1). The RT was performed as described in Material and Methods.

The cDNA for segment A was amplified in two overlapping PCR fragments (A1 and A2, overlapping by 639 nt) with Phusion High-Fidelity DNA Polymerase 100 U (2 U/μL) (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. PCR’s conditions were: 98 °C for 30 s, followed by 32 cycles of 98 °C for 10 s, 54 °C for 30 s, 72 °C for 3 min and a final extension of 72 °C for 10 min. The recombination primers used to amplify the A1 and A2 (IBDV A-F and VP2R; VP2F and IBDV A-R, respectively) and B (IBDV B-F and IBDV B-R) sequences are described in Table 1. The cDNA for segment B was amplified in one fragment using the IBDV B-F and IBDV B-R primers and the same RT and PCR conditions described for segment A.

Plasmid constructs
The construction of the plasmid pJG-CMV-HDR has previously made by Gil and Silva (unpublished data), and was made by insertion of cytomegalovirus (CMV) promoter, a poly linker region and the hepatitis delta virus (HDV) ribozyme sequence into pB42AD vector (OriGene, Rockville, MD, USA) (data not shown). The pJG-CMV-HDR plasmid has coding sequence for tryptophan (trp) amino acid as selectable marker gene for transformed cells selection on medium without trp. For cloning of IBDV infectious clone, the vector pJG-CMV-HDR was linearized with XhoI nuclease (Promega, USA) according to the manufacturer’s protocol. The PCR products (A1, A2 and B) containing homologous ends sequences with linearized vector could be directly cloned downstream of the CMV promoter of the vector pJG-CMV-HDR to produce the recombinant plasmids pJG-IBDV-Br-A and pJG-IBDV-Br-B, respectively. All clones were generated by homologous recombination in Saccharomyces cerevisiae strain RFY206 (MATα, trp1, ura3-52, his3-200, leu2-3, lys2-68, trp1::hisG). The selections of positive colonies (recombinated plasmid) were performed using Yeast Nitrogen Base (YNB) without trp (YNB - trp). The transformation of competent RFY206 was made by lithium acetate procedure as described previously (Sambrook and Russell, 2001). Positive colonies were grown YNB-trp and plasmid DNA extraction was performed using QIAprep Miniprep Kit according to manufacturer’s instruction (Qiagen, Valencia, CA, USA). Colonies were screened by PCR to confirm the cloning. The PCR was performed with recombinant primers described in Table 1 (IBDV A-F/ IBDV A-R and IBDV B-F/ IBDV B-R) to pJG-IBDV-Br-A and pJG-IBDV-Br-B, respectively. The PCR conditions were the same described in Material and Methods.

Escherichia coli electroporation
In order to amplify the DNA after homologous recombination, the purified yeast DNA was used to transform competent Escherichia coli strain DH10B (Invitrogen, Carlsbad,
CA, USA). pJG-IBDV-Br-A and pJG-IBDV-Br-B DNA plasmids were electroporated using 2 mm cuvettes on an ECM BTX electro cell manipulator 830™ (BTX Instrument Division, Holliston, MA, USA) with the following settings: 2.75 kV, 99 μs and 5 pulses with 1 s interval. Individual colonies were grown at 37 °C overnight in LB medium with ampicillin. Plasmid DNA was then purified using a QIAGEN Plasmid Midi Kit (Qiagen, Valencia, CA, USA) according manufacturer’s instructions.

**Transfection of CEF cells**

CEF cells were seeded at 7 x 10^5 cells/well of a 6-well tissue culture plate and cultured overnight. Cells were co-transfected with pJG-IBDV-Br-A and pJG-IBDV-Br-B using the PolyFect Transfection Reagent according to the manufacturer’s protocol (Qiagen, Valencia, CA, USA). pJG-IBDV-Br-A and pJG-IBDV-Br-B DNA used in the transfection, we included a negative control (pJG-IBDV-Br-A). The negative control was performed with only the transfection of the pJG-IBDV-Br-A.

**Determination of IBDV cDNA infectious clone (IC-IBDV-Br) infectivity**

At 5 days post-transfection, the cell supernatant was collected (Passage 0; P0), and used to re-infect freshly prepared CEF cells for five additional serial passages (P1-P5). Verification of generated and infectious IC-IBDV-Br was made by cytopathic effect (CPE) in CEF cells and amplification of the A segment using extracted vRNA from cell supernatant P5. To avoid amplification of residual plasmid DNA used in the transfection, we included a negative control reaction without the reverse transcriptase.

**Phenotypic Characterization**

**Plaque Assay**

CEF cells were seeded in 6-well tissue culture plates (7 x 10^5 cells/well) and incubated overnight. Monolayers were infected with serial dilutions of the parental virus (Wt-IBDV-Br) or IC-IBDV-Br (10^-1 to 10^-9). After 1 h absorption, the cells were washed once with PBS and overlaid with F10/199 medium containing 1% agarose and 5% FBS. After 3 days, cell supernatants were collected (Passage 0; P0), and used to re-infect freshly prepared CEF cells for five additional serial passages (P1-P5). To determine the genetic stability of the IC-IBDV-Br, its genome was compared to Wt-IBDV-Br clone by genomic alignment (Ape-A plasmid Editor v1.10.4).

**Results**

**Isolation and identification of the Wt-IBDV-Br**

The analysis of chicken embryo survival after virus inoculation is presented in Table 2. The dead embryos on the fourth day had a lower body development compared to control embryos, beyond bleeding in the skin, muscle and internal organs. All embryos had at least one of these lesions (data not shown). The CPE observed during all passages in CEF indicates that the Wt-IBDV-Br replicate efficiently in cell culture. The comparison Wt-IBDV-Br VP2 region vs. NCBI sequences confirmed the virus identity and identified the sequences in access number AJ310185.1 (access date 08/15/2012) as the sequences of greatest homology (E value 0.0 and Identity 99%).

**Construction of the full-length cDNA clone of IBDV genome**

To build the reverse genetics system, we have amplified and cloned the segment A and B. The segment A was amplified in two overlapping PCR fragments (A1 and A2) and segment B in one PCR product (Figure 1A). The amplified A1 (1173 bp), A2 (2789 bp) and B (2827 bp) were then assembled by homologous recombination into the pJG-CMV-HDR to produce pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids, respectively. The pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids were confirmed by full-length PCR (Figure 1B).

The comparison pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids vs. NCBI sequences identified the AJ310185.1 and EU162095.1 sequences (access date 08/15/2012) as the most homology to pJG-IBDV-Br-A (E value 0.0 and Identity 99%) and pJG-IBDV-Br-B (E value 0.0 and Identity 99%) respectively. Analysis of pJG-IBDV-Br-A and pJG-IBDV-Br-B vs. NCBI sequences are shown in Table 3.

**Identification of generated IBDV virus from plasmid constructs**

The IC-IBDV-Br identification was first performed by visualization of the CPE in cell culture. An additional verification was conducted by the segment A amplification (3260 bp) by RT-PCR of IC-IBDV-Br infected cells (Figure 2).

**Phenotypic characterization in cell culture**

The plaque assay confirmed the efficient replication of rescued virus in cell culture and shown morphology very similar to the Wt-IBDV-Br (Figure 3). The IC-IBDV-Br also exhibited growth kinetics similar to Wt-IBDV-Br (Figure 4).
Genetic stability of the recovered virus

IC-IBDV-Br was propagated in CEF cells (up to 5 passages) and its genome sequence was compared to pJG-IBDV-Br-A and pJG-IBDV-Br-B sequences. The IC-IBDV-Br sequences were submitted to GenBank (accession number KC603937 and KC603936 to A and B segments, respectively). The analysis of IC-IBDV-Br genome vs. pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids is showed in Table 4.

Table 2 - Analysis of embryo after inoculation with samples collected from chickens with suspected IBDV infection.

| Chicken | Samplea | 1° | 2° | 3° | 4° | 5° | 6° | 7° |
|---------|---------|----|----|----|----|----|----|----|
| 1       | BF-1    | -  | -  | -  | -  | -  | -  | -  |
|         | BF-2    | -  | -  | -  | -  | -  | -  | -  |
|         | Spleen-1| -  | -  | +  | -  | -  | -  | -  |
|         | Spleen-2| -  | -  | -  | -  | -  | -  | -  |
| 2       | BF-1    | +  | -  | -  | -  | -  | -  | -  |
|         | BF-2    | -  | -  | +  | -  | -  | -  | -  |
|         | Spleen-1| +  | -  | -  | -  | -  | -  | -  |
|         | Spleen-2| -  | -  | -  | -  | -  | -  | -  |
| 3       | BF-1    | -  | -  | -  | +  | -  | -  | -  |
|         | BF-2    | -  | -  | -  | -  | -  | -  | +  |
|         | Spleen-1| +  | -  | -  | -  | -  | -  | -  |
|         | Spleen-2| -  | -  | -  | -  | -  | -  | -  |
| 4       | BF-1    | +  | -  | -  | +  | -  | -  | -  |
|         | BF-2    | -  | -  | -  | -  | -  | -  | +  |
|         | Spleen-1| -  | -  | -  | -  | -  | -  | -  |
|         | Spleen-2| -  | -  | -  | -  | -  | -  | -  |
| 5       | BF-1    | -  | -  | -  | -  | +  | -  | -  |
|         | BF-2    | +  | -  | -  | -  | -  | -  | -  |
|         | Spleen-1| +  | -  | -  | -  | -  | -  | -  |
|         | Spleen-2| -  | -  | -  | -  | -  | -  | +  |

*aSamples collected from five commercial chickens with 26-27 days old.
bSamples were inoculated in chorioallantoic membrane of Specific Pathogenic Free eggs with nine days old. After inoculation, eggs were monitored during seven days by ovoscopy looking for embryo mortality.
BF: bursa of Fabricius processed in duplicate (BF-1, BF-2).
*Spleen processed in duplicate (Spleen-1, Spleen-2).
+: dead embryos, -: embryo survival.

Figure 1 - (A) Amplification of segment A and B using RNA extracted from CEF supernatant inoculated with Wt-IBDV-Br. Segment A was amplified in two overlapping PCR fragments A1 and A2 (1 and 2) with 1173 and 2789 bp, respectively. Segment B was amplified in one PCR product with 2.827 bp (3); (B) Full-length PCR to confirm the segment A (lane 1, 3260 bp) and B (lane 2, 2827 bp) cloning in pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids. M1: 1-kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA); M2: Lambda DNA/HindIII marker (Invitrogen, Carlsbad, CA, USA).

Genetic stability of the recovered virus

IC-IBDV-Br was propagated in CEF cells (up to 5 passages) and its genome sequence was compared to pJG-IBDV-Br-A and pJG-IBDV-Br-B sequences. The IC-IBDV-Br sequences were submitted to GenBank (accession number KC603937 and KC603936 to A and B segments, respectively). The analysis of IC-IBDV-Br genome vs. pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids is showed in Table 4.
IBDV is a highly contagious pathogen responsible for economic losses in the poultry industry worldwide. The lesions observed after inoculations of Wt-IBDV-Br in SPF eggs, such as hemorrhage and delayed embryonic development, were similar to the lesions observed by Perera et al. (2002) and similar to first descript for IBDV in Gumboro, USA (Cosgrove, 1962). In addition to suggestive lesions of IBDV infection, the sequence analysis of the VP2 region of isolated virus confirmed the IBDV identity.

| Genome region | Segment A | Segment B |
|---------------|-----------|-----------|
|               | pJG-IBDV-Br-A | AJ310185.1 | pJG-IBDV-Br-B | EU162095.1 |
|               | nt<sup>a</sup> | nt<sup>a</sup> | f<sup>b</sup> | f<sup>b</sup> |
| UTR (nt)<sup>c</sup> | 45 | g | c | 41 | a | t |
| | 49 | g | a | |
| | 69 | a | g | |
| | 102 | c | t | |
| ORF (aa)<sup>d</sup> | 356 | Tyr | Tyr | 303 | Pro | Pro |
| | 649 | Tyr | Tyr | 942 | Ile | Ile |
| | 969 | Thr | Asn | 999 | Lys | Lys |
| | 1107 | Leu | Ser | 1404 | Ala | Ala |
| | 1377 | Ile | Thr | 1627 | Leu | Leu |
| | 1765 | Ala | Ala | 1720 | Val | Ile |
| | 2212 | Ala | Ala | |
| | 2235 | Lys | Arg | |
| | 2761 | Leu | Leu | |

<sup>a</sup>Nucleotide.
<sup>b</sup>Untranslated Region, mutations shows in nucleotides.
<sup>c</sup>Open reading frame, mutations shown in amino acids.
<sup>d</sup>Silent mutations.
<sup>e</sup>Amino acids with the same chemical function.
<sup>f</sup>Access number of the sequences of greatest homology with pJG-IBDV-Br-A and pJG-IBDV-Br-B plasmids (access date 08/15/2012).

**Discussion**

IBDV is a highly contagious pathogen responsible for economic losses in the poultry industry worldwide. The lesions observed after inoculations of Wt-IBDV-Br in SPF eggs, such as hemorrhage and delayed embryonic development, were similar to the lesions observed by Perera et al. (2002) and similar to first descript for IBDV in Gumboro, USA (Cosgrove, 1962). In addition to suggestive lesions of IBDV infection, the sequence analysis of the VP2 region of isolated virus confirmed the IBDV identity.

The comparison of the segment A in pJG-IBDV-Br-A plasmid vs. AJ310185.1 (NCBI) showed that all observed changes in open reading frame were silent or with aa of the same chemical function, except for the 969, 1107, 1377 nt. The 969 and 1377 mutations changes were not observed in any GenBank sequences. However, the 1107 mutation changes already was found in PBG-98 strain, GenBank, access number D00868.1 (access date 10/15/12). The segment B changes in Wt-IBDV-Br clone (pJG-IBDV-Br-B plasmid) vs. EU162095.1 (NCBI) were all silent or with aa of the same chemical function. The comparison between the nucleotide sequence of pJG-IBDV-Br-A and pJG-
IBDV-Br-B plasmids and IC-IBDV-Br virus rescued showed changes of three and five nucleotide in segment A and B, respectively. Interestingly, all nucleotide changes into segment B in pJG-IBDV-Br-B plasmid vs. IC-IBDV-Br rescued virus were silent mutations or for aa of the same chemical function, and all these are also present in EU162095.1 (NCBI).

In order to rule out that the changes were not from the homologous recombination in yeast, the gene regions with these changes were sequenced from Wt-IBDV-Br virus, and it showed the same nucleotides of pJG-IBDV-Br-B plasmid (data not shown). This result supports the stability of virus genome during the cloning process by homologous recombination in yeast.

After the sequencing and genetic characterization of the Wt-IBDV-Br isolated, the viral reverse genetics system was constructed. This system is an important tool for investigations of the viral life cycle and biotechnology application (Gao et al., 2011). The reverse genetics system for IBDV has undergone changes over time. The first reverse genetics system for IBDV was possible by transfection into Vero cells with in vitro transcribed RNA of both segments (Mundt and Vakharia, 1996). An improved technique was followed by transfection of viral cDNA cloned downstream of the T7 (Boot et al., 1999) or CMV promoter (Lim et al., 1999).

The comparison between the two strategies showed the latter as more efficient for generation of IBDV viral particles (Boot et al., 2001). Additional improvements were also followed by addition of ribozyme sequences at both ends (5 and 3) (Qi et al., 2007) or only at the 3 of the cloned DNA (Ben Abdeljelil et al., 2008). Nowadays, the transfection of cloned viral cDNA has been used to evaluate the viral protein influence on IBDV virulence and more recently to development of recombinant viruses for vaccine purposes (Gao et al., 2011).
However, all assembly of the plasmids used to generate IBDV particles involves multiple rounds of amplification and need of in vitro ligation and restriction sites. Thus, we report the first reverse genetics system for IBDV constructed from homologous recombination in yeast. This method has been described in the literature for the genetic manipulation of other viruses, such as Yellow Fever (Queiroz et al., 2013) and HIV-1 (Marozsan and Arts, 2003) and it has shown to be efficient and easier to perform compared to the use of restriction enzymes and in vitro ligation.

The genetic stable and the similar phenotypes between IC-IBDV-Br and WI-IBDV-Br confirm the ability of the homologous recombination system in yeast to maintain the characteristics of the parental virus from which the reverse genetics system was built on. The development of this biotechnological resource will help to improve in depth the knowledge on IBDV virology and may be used in vaccines development.

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