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In vitro reassortment between Infectious Pancreatic Necrosis Virus (IPNV) strains: The mechanisms involved and its effect on virulence

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ABSTRACT

Reassortment is one of the main mechanisms of evolution in dsRNA viruses with segmented genomes. It contributes to generate genetic diversity and plays an important role in the emergence and spread of new strains with altered virulence. Natural reassorment has been demonstrated among infectious pancreatic necrosis-like viruses (genus *Aquabirnavirus, Birnaviridae*). In the present study, coinfections between different viral strains, and genome sequencing by the Sanger and Illumina methods were applied to analyze the frequency of reassortment of this virus *in vitro*, the possible mechanisms involved, and its effect on virulence. Results have demonstrated that reassortment is a cell-dependent and non-random process, probably through differential expression of the different mRNA classes in the ribosomes of a specific cell, and by specific associations between the components to construct the ribonucleoprotein (RNP) complexes and/or RNP cross-inhibition. However, the precise mechanisms involved, known in other viruses, still remain to be demonstrated in birnaviruses.

1. Introduction

Infectious Pancreatic Necrosis Virus (IPNV), a member of the genus *Aquabirnavirus* (family *Birnaviridae)*, is the etiological agent of a highly contagious disease that causes high mortalities, especially in young farmed salmonids. This disease is endemic in different areas and the virus has been isolated from a wide range of fish species.

IPNV is a non-enveloped icosahedral virus, with a bi-segmented ds RNA (Lightner and Post, 1969; Cohen et al., 1973; Dobos, 1977). Segment A contains two partially overlapping open reading frames (ORFs): the smaller one is located at the 5′ end and encodes the VP5 protein (Duncan et al., 1987; Magyar and Dobos, 1994; Dobos, 1995a), and the larger encodes a polyprotein, which is processed by the protease activity of VP4, yielding VP2, VP3 and VP4. VP2 is the major structural protein and is related to virulence (Heppell et al., 1995; Blake et al., 2001; Santi et al., 2004). VP3 is involved in virus assembly and genome packaging (Pedersen et al., 2007). Segment B contains only one ORF that encodes VP1, the viral polymerase (Duncan et al., 1991; Dobos, 1995b).

The high diversity (both genomic and antigenic) of this viral group is well known, and it has been recently demonstrated to be even higher due to the existence of natural reassortment. Reassortants were first reported by Romero-Brey et al. (2009) when analyzing the genomic diversity of aquabirnavirus isolates among natural fish populations from the Flemish Cap. Since then, a large number of reassortant strains have been detected in different locations (Cutrín et al., 2010; Lago et al., 2014; Moreno et al., 2014). This phenomenon has also been reported in infectious bursal disease virus (IBDV), a virus of another genus of the *Birnaviridae* family (g. *Avibirnavirus*) (Wei et al., 2008; Ksanga et al., 2013; Lu et al., 2015), and is also known in viruses from other viral groups, such as hantavirus, Lassa virus and tenuiviruses, with low rates of reassortment, and cystoviruses, rotavirus A and influenza A virus, with higher rates (Simon-Loriere and Holmes, 2011).

Reassortment is one of the main mechanisms of evolution in dsRNA viruses (Wei et al., 2006) and, together with sequential point mutations and recombination, is one of the key factors shaping the structure of genes and genomes, which contributes to genetic diversity (Devold et al., 2006). Replication of the two variants of a virus in the same target cell leads to the exchange of the genomic RNA segments and generates new reassortant strains. Two possible mechanisms of reassortment have been suggested: a random incorporation of segments during the replication cycle, and a selective incorporation which requires genomic signals (Pérez-Losada et al., 2014). Although, the mechanisms of reassortment still remain partially unknown, evidence exists supporting the second hypothesis.

It has been postulated that reassortment facilitates cross-species transmission and allows viruses to acquire new antigenic combinations that may assist in the process of entering a new host (Pérez-Losada et al., 2014). Furthermore, it plays an important role in the emergence and spread of new strains with altered antigenicity and/or pathogeni-

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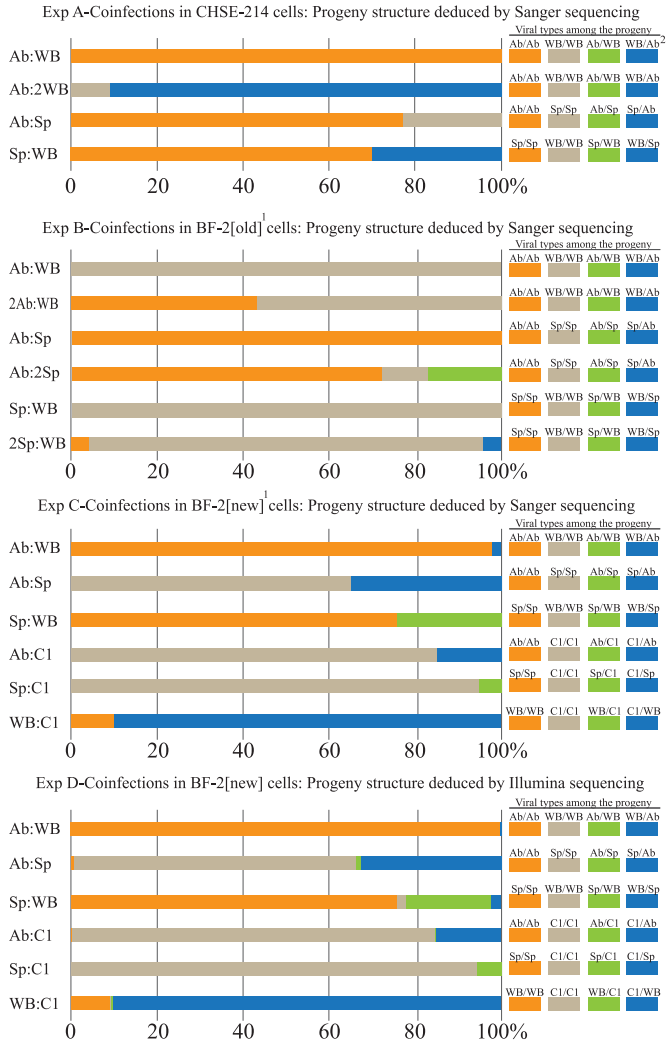

Fig. 1. Distribution of viral types among the progeny after coinfection with 2 IPNV strains: Cell cultures were coinfected with two parental types and, after total destruction of the monolayer, the crude virus was subjected to i) cloning by serial dilutions to sequence each clone (both segments) to determine the frequency of types among the progeny (experiments A to C; Sanger sequencing), or ii) sequencing by next generation sequencing (by the Illumina technology; experiment D). Coinfections were performed at ratios 1:1 (e.g. Ab:WB), 1:2 (e.g. Ab:2WB) or 2:1 (e.g. 2WB:Ab). 1- BF-2[old]: a cell line clone with more than 200 passages; BF-2[new]: new clone from the European Cell Culture Collection (ECACC). 2- Type of viral genome: Segment A/Segment B.

city (Ramig, 1997; Ohashi et al., 2004; Nikolakaki et al., 2005), and in the appearance of new phenotypes after the reassortment between a high pathogenic and an avirulent strain (Le Nouën et al., 2006; Wei et al., 2008; Kirsanovs et al., 2010; Coetzee et al., 2014; Park et al., 2014).

The implication of reassortment in the modulation of the virulence in aquabirnavirus was previously suggested by our group (Lago et al., 2010, 2013). In the present study, we have analyzed the frequency of reassortment in *in vitro* conditions, the possible mechanisms involved, and its effect on the virulence of the progeny.

2. Results

2.1. *Reassortment* in vitro

The results of the 4 sets of co-infection experiments are shown in Fig. 1 and Supplementary Table 1. In the co-infections performed in CHSE-214 at ratio 1:1, reassortment was achieved only when the wild types Sp and WB were used, obtaining 70% of clones of the type Sp and 30% of the reassortant WB/Sp. Co-infection between the Ab and Sp types yielded a progeny composed exclusively of the wild types: 77% of Ab and 23% of Sp; this coinfection was repeated ones, yielding similar results: 81% of Ab and 19% of Sp (results not shown in Fig. 1). The progeny from co-infection Ab:WB was exclusively constituted by the wild type Ab in a first assay (Fig. 1), and also in the second run (results not shown). When this co-infection was repeated at a ratio 1:2 (Ab/2WB), the wild type Ab disappeared from the progeny, and most of this (91%) was constituted by the reassortant WB/Ab, completed with a low percentage (9%) of the second wild type (WB).

In a second set of experiments, performed in BF-2[old] cells (viral clones sequenced by the Sanger method), reassortants were not detected among the progeny of co-infections performed at a ratio 1:1. Both 1:1 co-infections with the wild type WB (Ab:WB and Sp:WB) yielded progenies exclusively constituted by the WB type, and only the Sp type was detected in the co-infection Ab:Sp. Changing the ratio of the co-infection with Ab and WB to 2:1 (2Ab:WB), in order to favour the presumably less productive type, the percentage of WB among the progeny was reduced to 57%, and the type Ab represented 43%, but no reassortants could be detected. At a ratio 1:2, the co-infection with Ab and Sp (Ab:2Sp) yielded a progeny mostly constituted by the wild type Ab (as with ratio 1:1), but the percentage was reduced to 72%; the second wild type represented 11%, and 17% was constituted by the reassortant Ab/Sp. Most of the clones of the progeny from co-infection 2Sp/WB was of the WB type (92%); 4% was of the wild type Sp, and a reassortant WB/Sp was also represented 4%.

The co-infection assays performed at ratio 1:1 were repeated in the new cell line (BF-2[new] cells) in two sets of experiments (C and D) sequenced by the Sanger and Illumina methods, respectively. In both cases, more reassortants were detected, but it is remarkable that using the Illumina method, more types of virus were observed, and more reassortants detected. Since this method provides just the percentage of each type of segment present in the progeny, an algorithm [Supplementary File 1] had to be designed to predict the corresponding putatitive combinations of each type of virus, yielding a range of percentages, instead of single values. The co-infection Ab:WB yielded a progeny constituted almost exclusively by the wild type Ab (98% with Sanger and 99.6–99.6% with Illumina); a reassortant WB/Ab was hardly represented (2% and below 0.4%, respectively), and the presence of the other 2 types (WB/WB and Ab/WB) was negligible (≤0.01% with Illumina). Both co-infections with the Sp wild type yielded progenies with a majority of the Sp type; in the co-infection Ab:Sp, the reassortant Sp/Ab represented around 32–35% (35 with Sanger and 32–33% with Illumina), and the other 2 types (Ab/Sp and Ab) below 1.2% with Illumina (not detected by Sanger); in the co-infection Sp:WB, a reassortant Sp/WB represented between 19% and 24% (24%, Sanger; 19.4–22.2%, Illumina) of the progeny, and the presence of the other 2 types (WB/Sp and WB) was below 2.8% with Illumina (not detected by Sanger).

In both sets of experiments, a fourth reference strain was assayed against the other 3. When C1 was co-infecting with Ab or Sp, most of the progeny (around 84% and 94%, respectively) was constituted by the wild type C1; in the progeny from co-infection Ab:C1, a reassortant C1/Ab was present at around 15%, and the representation of the other 2 types was not detected by Sanger and negligible (below 0.7%) by Illumina; in the co-infection Sp:C1, only a second viral type was detected -the reassortant Sp/C1-, at around 5% by both sequencing methods. Interestingly, the co-infection WB:C1 yielded a progeny mostly constituted by the reassortant C1/WB (around 90%); the wild type C1 was absent or negligible and the WB type was poorly represented (around 9–10%).

2.2. *Statistical analysis*

Table 1 shows the statistical analysis performed to determine if bias