In vivo evidence for quasispecies distributions in the bovine respiratory syncytial virus genome

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Abstract

We analyzed the genetic evolution of bovine respiratory syncytial virus (BRSV) isolate W2-00131, from its isolation in bovine turbinate (BT) cells to its inoculation in calves. Results showed that the BRSV genomic region encoding the highly variable glycoprotein G remains genetically stable after virus isolation and over 10 serial infections in BT cells, as well as following experimental inoculation in calves. This remarkable genetic stability led us to examine the mutant spectrum of several populations derived from this field isolate. Sequence analysis of molecular clones revealed an important genetic heterogeneity in G coding region of each population, with mutation frequencies ranging from 6.8 to 10.1 10^{-4} substitutions/nucleotide. The non-synonymous mutations of the mutant spectrum mapped preferentially within the two variable antigenic regions of the ectodomain or close to the highly conserved domain. These results suggest that RSV populations may evolve as complex and dynamic mutant swarms, despite apparent genetic stability.

Bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV) are negative strand RNA viruses that belong to the genus *Pneumovirus* within the family *Paramyxoviridae*. These two closely related pneumoviruses are a common and important cause of lower respiratory tract illness in calves and young infants (Larsen, 2000). The antigenic and genetic diversity among independent isolates of HRSV has been extensively documented, and the existence of two major groups (A and B) has been clearly established as well as additional variability within each individual group (reviewed by Sullender, 2000; Cane, 2001; Zlateva *et al*., 2004; 2005). The most extensive differences were found on the attachment glycoprotein G, which differs by up to 45% in its aminoacid (aa) sequence between A and B groups (Johnson *et al*., 1987; Mufson *et al*., 1985). The extent of antigenic variation observed with BRSV is considerably less than for HRSV and the different subgroups identified may only represent variants of a single major antigenic group (Furze *et al*., 1994; Schrijver *et al*., 1996; Prozzi *et al*., 1997). While BRSV nucleotide sequence of the variable G gene does not exceed 11% between independent isolates (Larsen *et al*, 2000; Valarcher *et al*., 2000), this genetic variability may have biological implications such as escape from previous vaccine immunity, as suggested by a comparative phylogenetic study on the G, F and N genes (Valarcher *et al*., 2000).

Genetic variation is a hallmark of RNA viral pathogens. For RNA virus, mutation rates operating during RNA replication have been estimated to be in the range of 10^{-3} to 10^{-5} substitutions per nucleotide and round of copying (Drake, 1993; Drake & Holland 1999). The biochemical basis of the limited replication fidelity is the absence or the low efficiency of a 3' → 5' exonuclease proofreading activity in viral RNA-dependent RNA polymerases, together with lack of post replicative mismatch repair mechanisms (Domingo & Holland, 1997). As a consequence, populations of RNA viruses evolve as dynamic distributions of closely related mutant genomes that exist in equilibrium around a theoretical consensus sequence. Such mutants would provide evidence of quasispecies dynamics, implying the presence of a variant reservoir

for viral adaptation. Despite the biological significance of this population structure, no such analyses of mutant spectra have been reported for RSV to date. In the present work, we demonstrate that BRSV populations evolve, *in vitro* and *in vivo*, as complex and dynamics mutant swarms, despite apparent genetic stability.

The replicative environment provided by cell culture conditions may exert a selective pressure on replicating RNA viral populations (Domingo *et al*., 2001b). To test the genetic stability of BRSV upon virus isolation and propagation in cell culture, we analyzed the consensus nucleotide sequence of the highly variable glycoprotein G coding region of a natural population of BRSV at different stages of its evolutionary history (Fig. 1). BRSV W2-00131 was isolated from bronchoalveolar lavage (BAL) of a calf with distress respiratory syndrome (BAL-T) and further propagated in bovine turbinate cells (BT, ATCC CRL-1390) (Valarcher *et al*., 2001). To prove that multiple variants are produced *de novo* upon replication of a single BRSV genome, biological cloning was performed as follows: serial virus dilutions (order of 2) were used to infect BT cells in fresh agar MEM medium (Valarcher *et al*., 2001). After four days, one individual syncytium at the latest dilution was picked under microscopy and amplified for one round in BT cells. Two additional steps of cloning were performed using the same method. Finally a single syncytium 3C was picked and propagated in BT cells. Populations 3Cp3, 3Cp9 and 3Cp10 were isolated after 3, 9 and 10 passages of the clone 3C in BT cells respectively (Fig. 1). In addition, population 3Cp9 was used in experimental infection. For this, calves were reared in biocontainment facilities from birth to euthanasia, as prescribed by the guidelines of the UE Council on Animal Care (86/609/CEE). Calves were infected at 4-5 months of age with 10^7 PFU of virus by aerosolisation (UltraNeb 99nebulizer, DevilBiss). BAL-E and BAL-G populations were then isolated by BAL at days 7 and 9 post-infection from calf E and G respectively, as already described (Valarcher *et al.*, 2001).

To study the BRSV genetic stability, we analysed the consensus sequence of the G gene of viral populations replicated *in vitro* (3Cp3, 3Cp9, 3Cp10) and isolated *in vivo* (BAL-T, -E and –G). Genomic RNA extracted from viral populations was subjected to RT-PCR amplification (Valarcher *et al*., 1999). Five independent PCRs, from two reverse transcription steps, were performed on the entire BRSV G gene with primers G1 and G2 (positions 4682-4703 and 5501-5475, Gene Bank NC_001989) for 3Cp and BAL-T, or on partial G gene with primers VG1 and VG4 (positions 4835-4855 and 5376-5356, Gene Bank NC_001989) for BAL-E and BAL-G. DNA amplification was performed using the high fidelity ThermalAce DNA polymerase (Invitrogen) in a GeneAmp PCR System 9700 (PE Applied Biosystems) for 35 cycles (95 °C for 30s, 58 °C for 30s, 72 °C for 45s). RT-PCR controls were performed on RNA diluted 1/10 to ensure that stock RNAs were not in limited dilution. Sequence determination at glycoprotein G coding region revealed that the consensus viral genome found in BAL of calf T remains dominant following virus isolation and genetically stable over 10 serial infections in BT cells. Likewise, no modification of the G consensus sequence of BRSV W2-00131 populations was observed following virus replication in calves.

The genetic stability exhibited by the glycoprotein G led us to examine the mutant spectrum of the BRSV populations derived from this field isolate. For each population the previous RT-PCR products were cloned in the pCR4 Blunt TOPO plasmid (Zero blunt Topo PCR cloning kit; Invitrogen). From about 26 to 29 molecular clones were sequenced and analyzed (SeqMan 4 DNASTAR Applied Biosystem) for one population (five to six positive *E. coli* clones for each of the five-independent PCRs). The mutation frequency of 3Cp populations ranged from 6.8 to 9.9 x 10^{-4} substitutions per nucleotide (Table 1). A significant heterogeneity was also observed within BRSV populations replicating in the animal host with mutation frequencies that range from 7.5 to 10.1 x 10^{-4} substitutions per nucleotide. These mutation frequencies are near to those observed for highly variable RNA viruses, such as vesicular stomatitis virus and poliovirus

(Domingo & Holland, 1994). However, conservation among independent isolates often parallels conservation within mutant spectra (Domingo *et al.,* 2001a). Consequently, mutation frequencies obtained in this study could be overexpressed, since the G gene was the most highly variable. Confirmation or not would require sequencing of other BRSV genomic regions.

To determine the extent of artifactual misincorporations introduced by the high fidelity ThermalAce DNA polymerase during the amplification procedure, the BRSV G gene was cloned in the plasmid pGemT (Promega) under the control of the T7 RNA polymerase promoter. One clone was *in vitro* transcribed using the T7 RNA polymerase kit (Promega). After Dnase treatment, the RNA transcripts, diluted 1/1000, were subjected to the original RT-PCR procedure and the amplified products were subcloned in the pCR4 Blunt TOPO plasmid. The sequence of 25 clones from the G coding region yielded an error rate of $1.2\ 10^{-4}$ mutations per nucleotide. Since the error rate of the T7 RNA polymerase is about $2.9\ 10^{-5}$ (Remington *et al*., 1998), the final error rate of the system was calculated to be $0.9\ 10^{-4}$ mutations per nucleotide. The ANOVA and a multiple comparison test, with the bonferroni correction, revealed significant differences when each BRSV population frequency was compared with the system error rate. This indicated that the mutation frequencies observed cannot be the result of misincorporations during RT-PCR amplification of BRSV RNA.

The genetic heterogeneity derived from clone 3C suggests a constant generation of mutant genomes in the course of BRSV replication. To provide further evidence of the error-prone replication of BRSV genome, we analyzed the mutant spectrum of a virus population rBRSV, derived by transfection of a single infectious cDNA clone of ATue 51908 strain (Buccholz *et al.,* 1999) and kindly provided by Dr K.K. Conzelmann (Federal Research Center for Virus Diseases of Animals, Tübingen, Germany) after two passages in MDBK cells. Five additional passages of rBRSV in BT cells were performed before analysis. The rBRSV mutation frequency of 7.7×10^{-4} substitution per nucleotide for glycoprotein G coding region is in the range of previous mutation

frequencies for other isolates (Table 1). These results clearly indicate that multiple variants are produced *de novo* upon replication of a single viral genome. Recently, genetic variation of the consensus sequence of HRSV strains likely derived from one single clone was also demonstrated with mutation rates of 2.5 to 3.0 10^{-3} nt substitution/site/year (Trento *et al.,* 2006).

Several arguments support the fact that the great majority of mutations founded was present in the BRSV W2-00131 RNA populations. A large dominance of non-synonymous (*Nsyn*) over synonymous mutations was observed in all BRSV mutant spectra. These *Nsyn* mutations accumulate in all parts of the G protein ectodomain, preferentially but not significantly in the two mucin-like regions (Fig. 2). Variability within the G immunodominant central domain of the mutant spectrum was also observed in consensus sequences of BRSV published isolates (Prozzi *et al.*, 1997, Valarcher *et al*., 2000). However, it contrasts with observations made from HRSV sequences, which indicate that *Nsyn* mutations were specifically located in the mucin-like regions within subgroups (Cane *et al.,* 1991). In this study, *Nsyn* mutations preferentially mapped in antigenic regions of RSV G glycoprotein located in the C-terminal part of the protein (Rueda *et al*., 1991; 1995; Sullender, 1995; Melero *et al*., 1997) and in defined domains of the mucin-like and the central conserved regions (Lerch *et al*., 1990; Langedijk *et al*., 1996; Prozzi *et al*., 1997; Walsh *et al*., 1998). Selection of HRSV immune escape mutants was shown to be associated with such a single amino acid substitution in these antigenic domains (Garcia Barreno *et al*., 1990; Rueda *et al*., 1991; 1995; Martinez *et al*., 1997). However, for BRSV, Woelk and Holmes (2001) recently failed to detect positive selection in the G gene suggesting that some of the variability observed in the G protein was not a consequence of selection by the immune response. In this study, no major selective pressure was present, as corroborated by the identity of the G consensus sequences of all BRSV populations. Consequently, the high level of aa variation we found in the mutant spectrum more probably reflects reduced constraints for variation at glycoprotein G in the ectodomain. The fact that many of these aa changes were encountered in the consensus sequence

of independent isolates (Valarcher *et al.,* 2000) also indicates that these mutations are well tolerated and may not adversely affect protein function. Variable mechanisms observed for glycoprotein G variability also illustrate the capacity of this protein to accommodate multiple sequence changes (Melero *et al.,* 1997). Theses mechanisms include aa substitutions but also insertions/deletions, changes in the stop codon usage and frameshift mutations. Some of these situations were found in molecular clones of the BRSV populations studied, suggesting their effective presence in the mutant spectrum. Two BRSV populations (BAL-T and 3Cp10) contain one clone with a mutation in the stop codon (TAG 258 → glutamic acid), leading to a 6 aa longer protein. Differences in the length of the BRSV and HRSV G glycoproteins were demonstrated to result from use of such alternative stop codons (Garcia Barreno *et al*., 1990; Rueda *et al*., 1991; Zlateva *et al*., 2005). Remarkably, the number of deletions found in all but one population was high in this study (Table 1). Examination of the sequence context indicates that 85 % of the deletions included one or two nucleotides within short homopolymeric-A and -C tracts. Two poly-A tracts (nt 607-610 and 622-626 of G gene) correspond to poly-A runs which were described for HRSV to be very prone to polymerase errors, resulting in frame-shifts because of the insertion or deletion of adenosine residues (Cane *et al.,* 1993). Surprisingly, we also found two clones in populations 3Cp10 and BAL-T with a C deletion in one homopolymeric poly-C run (nt 467-471). This deletion introduced a frame-shift with a putative protein containing 155 aa of the N-part of the G glycoprotein followed by 55 aa of a new sequence. Viability of such clone was not demonstrated in this study but, for HRSV, escape mutants with frame-shift mutations in the consensus sequence generated by deletions in poly-A runs were clearly selected with antibody 63G (Garcia-Barreno *et al*., 1990). Some limited use of alternative frames and termination codons has also been found among HRSV isolates from patients (Sullender *et al*., 1991, Cane & Pringle, 1995).

Despite the presence of a large mutant spectrum, the G consensus sequences of each BRSV populations are identical, in agreement with results obtained after BRSV passages in cells or calves (Larsen *et al*., 1998; 2000). This does not exclude potential changes in the consensus sequence of other part of the BRSV genome. Results indicated the occurrence in BRSV populations of a dominant genomic sequence, which ranges from 48% to 68%, together with several variants. Except for one, all mutations were unique suggesting that despite mutant genomes arising at a high rate, each specific variant remain at a low frequency. The dominant and the consensus sequences were identical in all populations analysed and the values of Shannon entropy ranged from 0.38 to 0.52 with no significant differences among populations tested. Under those circumstances the mutant spectrum is probably nearly optimal, at the population equilibrium, suggesting that BRSV is well adapted to the biological environment. In this view, the 89 % or higher consensus sequence identities of G glycoprotein found among independent BRSV isolates (Valarcher *et al*., 2000), would be the result of negative selection on many newly arising mutants and convergence of consensus and average sequences (Eigen & Biebricher, 1988; Domingo *et al*., 2000). On the other hand, the 11% BRSV genetic variability is probably shaped by positive selective pressures of the replicative environment. Positive selection on G glycoprotein was clearly demonstrated to be associated with mutations in the G gene (Melero *et al.,* 1997; Sullender, 2000; Woelk & Holmes, 2001; Huang & Anderson, 2003).

In conclusion, this study shows the heterogeneous nature of BRSV genome populations and the low fidelity of their replication, despite genetic stability. Continuous generation of virus mutants is currently regarded as a key adaptative strategy of RNA viruses. Studies are underway to define the biological implications of BRSV viral heterogeneity.

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Figures

Fig. 1. Origin and passage history of BRSV populations.  = isolation from calves. ● = passages in BT; ■ = BRSV biological cloning by a three step end-point dilutions; ○ = selected populations for genetic studies.

Fig. 2. Representation of BRSV G aa substitutions of individual clones of 3Cp and BAL populations compared to the consensus sequence represented at the top. Notation above the sequence divides the protein into the intracellular domain (1-37), the transmembrane domain (38-66), the two variable (67-152; 194-257) and the conserved (153-193) regions of the ectodomain. Mutations observed at the same position in different populations are boxed in grey.

TABLE 1. Analysis of the mutant spectrum of BRSV populations

BRSV population	Clones (n)	Nucleotides sequenced *	Mutation frequency †	Ts‡	Tv‡	D‡	I‡	Syn/ Nsyn	Shannon entropy§
3Cp3	26	17,227	9.9×10^{-4}	12	5	0	0	5/12	0.56
3Cp9	27	20,777	7.7×10^{-4}	10	4	2	0	3/11	0.60
3Cp10	26	18,969	6.9×10^{-4}	9	3	1	0	4/6	0.51
BLA-T	26	19,844	10.1×10^{-4}	14	3	3	0	6/11	0.58
BLA-E	28	13,065	7.6×10^{-4}	2	4	4	0	2/4	0.44
BLA-G	28	13,319	7.5×10^{-4}	4	3	3	0	1/5	0.40
rBRSV	29	22,188	7.7×10^{-4}	10	5	1	1	6/9	0.43
pGEMT7-G	25	16,394	0.9×10^{-4}	2	0	0	0	3/0	0.11

* Total number of nucleotides sequenced.
† Minimum mutation frequency (number of different mutations found divided by the total number of nucleotides sequenced; Domingo *et al.,* 2000). Mutation frequency is expressed as substitutions per nucleotide**.**
‡ Ts (transitions); Tv (transversions); D (deletions); I (insertions); Syn (synonymous or silent mutations); Nsyn (nonsynonymous mutations)
§ The normalized Shannon entropy was calculated as $S_n = [\Sigma_i (p_i \times \ln p_i)]/\ln N$, where p_i is the frequency of each sequence and N is the total number of sequences.

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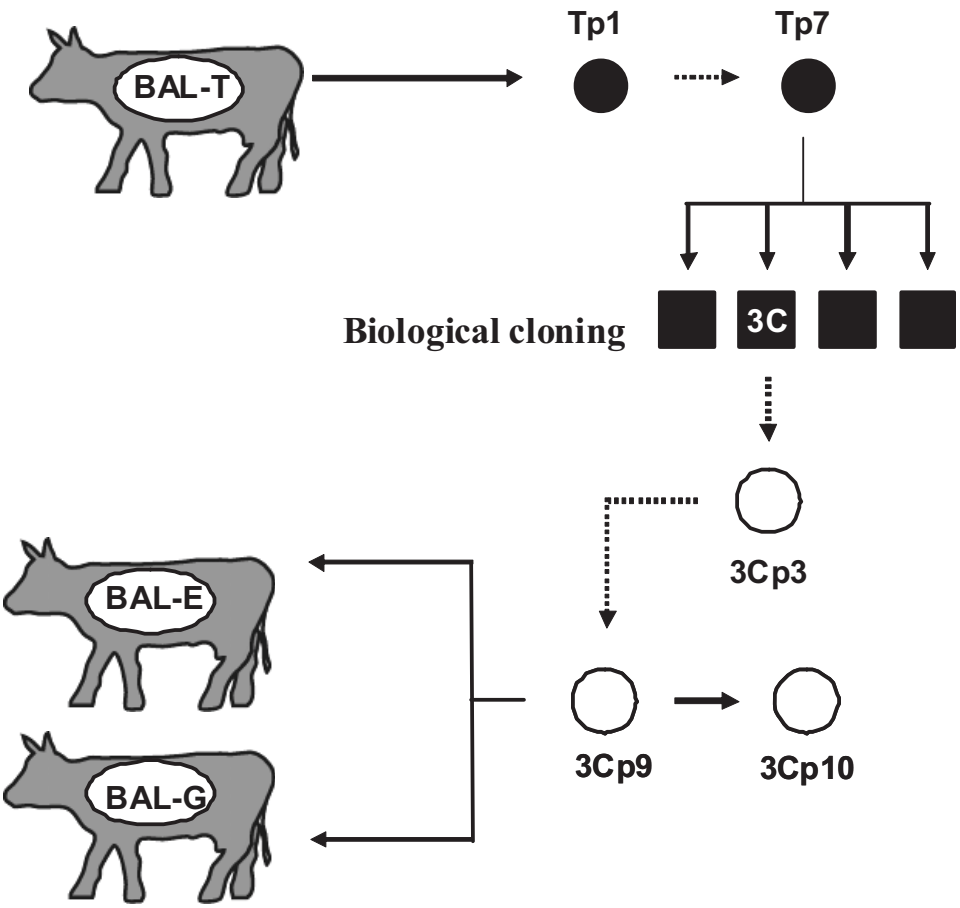

JGV 82668, Figure 1, Deplanche et al.