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AVIAN METAPNEUMOVIRUS REVERSE GENETICS DEVELOPMENTS AND THEIR EFFECT ON THE PERFORMANCE OF RECOMBINANT LIVE VACCINES

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1. INTRODUCTION

Avian metapneumovirus (AMPV) is an enveloped negative sense single stranded RNA virus, which is a major endemic respiratory pathogen of global domestic poultry. The virus causes acute respiratory tract infection in turkeys characterized by sneezing, tracheal râles, swollen sinuses and nasal discharge (Naylor and Jones., 1993). Infection of chickens results in a drop in egg production from laying birds and can be associated with swollen head syndrome (SHS) (Cook, 2000). Four subtypes of AMPV have been recognized worldwide: A, B, C and D. Subtypes A and B have now been reported in most countries worldwide, whilst subtype C has only been reported in the USA (Seal, 1998), France (Toquin et al., 2006), China (Sun et al., 2014; Wei et al., 2013) and in one case in Korea (Lee et al., 2007). Subtype D has only been reported in France (Bayon-Auboyer et al., 2000). Reverse genetic (RG) techniques have been applied to subtype A (Naylor et al., 2004) and C (Govindarajan et al., 2005); several reports have investigated the effects of single and multiple genomic mutations and gene deletions (Ling et al., 2008; Naylor et al., 2004) or insertions (Falchieri et al., 2013) on viral biology. A subtype B RG system has not been yet developed. This subtype is distributed worldwide and growing field evidence suggests it to be more able to infect commercial chickens compared to subtype A. For these reasons it would be convenient to have a RG system available also for B viruses. The aims of this study was to developed a RG system for AMPV subtype B and gain a better understanding of the viral capacity to accept and express heterologous extra sequences in order to developed effective AMPV recombinant vaccines.

In chapter 4 a comparison of subtype A and B viruses was performed to assess whether subtype A RG components could be partially or fully substituted. AMPV subtype A and B gene end sequences, as well as several leader and trailer sequences

were obtained. After comparing these data, reported gene start sequences and protein sequences, it was concluded that subtype B genome copies would be likely to be rescued by a subtype A support system. Individual subtype A components were substituted with subtype B components. A fully subtype B RG system was obtained using an advance cloning plasmid, and proved that all subtype specific components could be freely exchanged between A and B systems.

In chapter 5 was assessed the ability of subtype B to accept and express foreign genes, specifically spike (S1) and nucleocapsid (N) genes of infectious bronchitis virus (IBV). Recombinant viruses had been recovered by RG and proved to be able to express the inserted genes efficiently and to be stable during passage *in vitro*. Subsequently AMPV-B/IBV recombinants were tested as candidate vaccines by eye-drop inoculation of one-day-old chickens and challenged with IBV.

Chapter 6 investigates how to increase the protection induce by subtypes the recombinants. In a first study the genes involved in the replication process were modified to increase the replication *in vivo*. At the same time, the Interleukin 18 (IL-18) was added in the recombinants: IL-18 is known to play an important role in the inflammatory reaction in chickens. Viruses have been recovered and challenged *in vivo* against IBV.

In chapter 7 is described the attempts to develop viruses able to express multiple IBV proteins. The matrix (M) sequence of IBV was added in construct containing the S1 or the N genes. At the same time the exogenous genes were inserted in different positions along the AMPV genome sequence. Several constructs containing up to 3 exogenous genes were obtained.

2. LITERATURE REVIEW

2.1 AVIAN METAPNEUMOVIRUS LITERATURE REVIEW

2.1.1 AETIOLOGY

Avian metapneumovirus (AMPV) belongs to the *Metapneumovirus* genus; Metapneumoviruses are part of the subfamily *Pneumovirinae* within the *Paramyxoviridae* family, including single stranded, negative sense RNA, and enveloped viruses (Pringle, 1998; Van den Hoogen et al., 2001; Van Regenmortle et al., 2000). Human metapneumovirus (HMPV) it's the only other virus belonging to this genus.

2.1.1.1 Morphology

The virus can be seen by electronic microscopy; the viral particles appear pleomorphic, with shape ranging from spherical to filamentous. The viral particles size is also variable, ranging from 40 to 500nm. The nucleocapsid is characterized by a helical shape and on the envelope surface projections of about 13 – 14 nm are clearly distinguishable (Baxter-Jones et al, 1987; Buys et al., 1989; Collins et al., 1986; Cook et al., 2002; Giraud et al., 1986; Gough et al., 1989; McDougall et al., 1986; Wyeth et al., 1986).

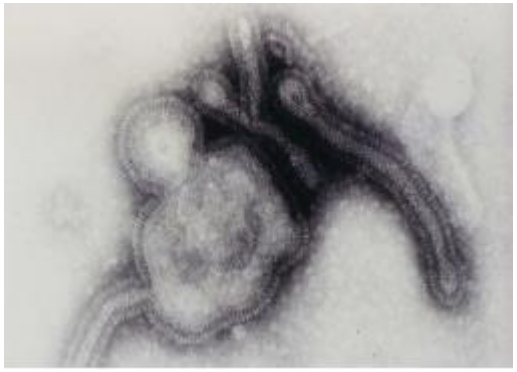


Figura 2.1 AMPV observed using electronic microscopy

2.1.1.2 Genome

AMPV is characterised by a negative sense single stranded RNA genome of about 13000 - 14000 nucleotides (Randhawa et al., 1997). The genome encodes for 8 genes: these 8 genes are translated in at least 9 proteins. The order of the gene from the 3' end to the 5' end is the following: N, P, M, F, M2 (including two overlapping open reading frames), SH, G and L (Easton et al., 2004; Ling et al., 1997). Every gene is flanked by a transcriptional start sequence and a transcriptional stop sequence, and between each transcriptional unit there are intergenic untranslated regions. Both the 3' end (leader) and the 5' end (trailer) of the genome show a complementary untranslated sequence of about 40 bases: these sequences contain promoters and are involved in the processes of transcription, replication and packaging (Ling et al., 2008; Wheelan et al., 2004).

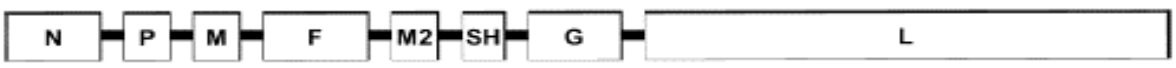


Figure 2.2 AMPV genome

2.1.1.3 Proteins

The 8 genes encode for 9 proteins. The Nucleocapside protein (N) forms the nucleocapside and it joins to the genome, being responsible for the helicoidal structure of the RNA (Easton et al., 2004). The phosphoprotein (P), together with the RNA dependent Polymerase (L) protein, forms the ribonuclear complex (RNP). Those two protein are involved in the processes of genome replication and genes transcription (Broor and Baraj, 2007). The Matrix (M) protein is situated in the inner envelope surface, anchoring the nucleocapside to the lipidic membrane. In contrast with the other genes, the Matrix 2 gene (M2) encodes for two different proteins: M2-1 protein, which seems to behave as a transcription elongation factor and M2-2 protein which is thought to act in the transition from the replicative phase to the assembly phase of the virion before the release of the latest from the host cell surface. The remaining three proteins are the glycoproteins of the envelope: the small hydrophobic protein (SH) is an integral membrane polypeptide; however its function is poorly understood. The Fusion (F) and the attachment (G) proteins, located on the external part of the envelope, are recognised as the major antigenic determinants of the virus (Broor and Baraj, 2007).

2.1.1.4 Virus attachment, transcription and replication

The protein involved in the attachment of the virus to the host cell receptors is the G protein. Once the virus is attached to the host cell surface, the F protein enables the fusion of the envelope with the cell membrane, leading to the release of the nucleocapside into the cytoplasm (Easton et al., 2004). AMPV, as all negative stranded RNA virus, needs a ribonuclear complex (RNP) both for the transcription and the replication: thus, to initiate an infectious cycle, the viral genome is incorporated in the nucleoprotein (N) and linked with the RNA dependent

Polymerase (L) and the phosphoprotein (P), that acts as cofactor. The polymerase enter the genome at the 3' end; the synthesis of the positive sense mRNA starts at the first transcription start sequence and stop at the first transcription stop sequence. At this stage the polymerase molecules can either move along the genome, beginning to transcribe a new gene binding at the following transcription start, or dissociate from the RNA and rebind to the 3' end, beginning the synthesis of the first mRNA. This mechanism applies to every transcription stop along the whole genome, therefore at every junction the polymerase can dissociate from the genome. The obvious consequence of the process is a gradually decrease in the mRNA production, moving from the 3' end to the 5' end (Dimmock et al., 2007). To a major mRNA synthesis correspond a major protein production.

To generate the positive sense copy of the genome the RNA dependent Polymerase must ignore the transcription start and stop flanking each gene, but the mechanism behind this behaviour of the L protein is still not clear. One hypothesis, suggested by studies on human respiratory syncytial virus (HRSV), is that the concentration of the N protein in the cytoplasm may play a role in the regulation of the process (Fearns et al., 1997). The positive sense copy of the genome is then used as template for a new negative sense RNA full genome. The new synthetized negative sense RNA genome forms a new RNP together with N, P and L proteins. The assembly process is led by the M proteins: this protein interacts with the RNP and the surface proteins, SH, G and F, which after synthesis have been inserted into the cell membrane; these last interactions, in particular, results in virions budding from the cell surface. (Easton et al., 2004).

2.1.1.5 Chemical and Physical properties

AMPV is stable in a pH range between 3 and 9, it's inactivated at 56°C in 30 minutes and it's sensible to lipoid solvent as ether and clorophorm (Collins et al., 1986). Aldehydes, alcohols, phenols and organic acids inactivate the virus (Hafez and Arns, 1991). AMPV is resistant to drying for 7 days but could survive for several days in turkey litter at different temperatures (Velayudhan et al., 2003). Autoclave, microwaving and high-pressure treatment are able to inactivate the virus.

2.1.1.6 Strain classification

Four subtypes of AMPV have been recognized worldwide: A, B, C and D. Most of the detection in Western Europe have involved subtypes A and B, with the exception of a French strains, isolated in 1985, resulted in the identification of subtype D (Bayon-Auboyer et al., 2000; Collins et al., 1993). In North America in 1997 was isolated a strain identified as subtype C (Seal, 2000). The first differentiations were performed using serological test (ELISA, seroneutralization, immunofluorescence), but now a day the sequencing of the F and the G genes is used (Collins et al., 1993; Naylor et al., 1998; Seal et al., 2000).

2.1.1.7 Nucleotide and amino acid identity

Subtypes A and B show a nucleotide identity about 56-61% whereas within subtypes it is 97-99% (Lwamba et al., 2005). Subtype C isolates have been reported to share 89-94% nucleotide identity, compared with 60-65% with subtypes A and B (Shin et al., 2002). A recently full genome sequence of the subtype D isolate, reveal that it is more related to subtype A and B rather than C (Brown et al., 2014).

The predicted amino acid (aa) sequence confirms that subtypes A, B and D are closer than subtype C. N, P, M and F are the most conserved sequences, with an aa identity up to 90% between subtypes A and B and about 50-70% between those two subtypes and subtype C (Jacobs et al., 2005;Li et al., 1996; Naylor et al., 1998; Randhawa et al., 1996; Seal, 1998; Seal et al., 2000; Shin et al., 2002). The M2:1 protein is very conserved in all the subtypes, with identities ranging from the 64% to the 89%, while the M2:2 is more variable: A and B type share the 71% of their aa sequence, while the percentage of identity decrease to the 20% when compare to the C type (Dar et al., 2003; Jacob et al., 2005). High variability has been observed in the SH and the G proteins where the identity is of 47% and 38% respectively between subtypes A and B and only 18% and 15% for subtype C in comparison with the other two subtypes (Govindarjan et al, 2004; Lwamba et al., 2005). The L gene share a 85% of identity between subtype A and B, while it decrease to 62.5% when compare with the C type L aa sequence (Lwamba et al., 2005; Sugiyama et al., 2010)

2.1.2 EPIDEMIOLOGY

2.1.2.1 Host

The natural hosts of AMPV are turkeys and chickens, with the first specie considered the most susceptible, as well as the first specie in which was observed the disease, firstly called Turkeys Rhinotracheitis (TRT) (Buys and Du Preez, 1980; Gough and Jones, 2008). Guinea fowls and pheasants are susceptible to the infection, as suggested by field evidences and experimental infection studies (Catelli et al., 2001; Gough el at, 1988; Horner et al., 2003; Laconi et al., 2014). Pigeons, ducks and geese seem resistant to the infection due to AMPV subtypes A and B (Gough et al., 1988); however some field studies carried on in North America

showed a low level of sensitivity to subtype C (Shin et al., 2000a; Turpin et al., 2008), as confirmed by experimental infection studies (Toquin et al., 2006a; Toquin et al., 2006b). Farmed ostriches in Zimbabwe were found serologically positive to AMPV, as well as some birds imported in Italy from Africa (Cadman et al., 1994; Capua, 1998). In general, wild species have been proven to be sensitive to AMPV subtype C, while the role of the other subtypes in the wild species is still not fully understood (Bennet el al., 2002; Bennet et al., 2004; Heffels-Redman et al., 1998; Turpin et al., 2008).

2.1.2.2 Distribution

AMPV has been detected worldwide, with the exception of the Australian continent (Bell et al., 1990). The disease has been seen for the first time in turkey farms in South Africa in the late '70's (Buys and Du Preez, 1980). Since then the virus spread rapidly in Europe and the first detection has been done in France in early '80s (Andral et al., 1985). In the following years the virus has been detected in others European countries: Germany (Hafez and Woernle, 1989), Spain and Italy (Fabris and D'aprile, 1990), Hungary (Lantos, 1990), Croatia (Bidin et al., 1990), Austria (Polland et al., 1992), Poland (Minta et al., 1995), Sweden (Engstroom et al., 2000) and Russia (Botchkov et al., 2002). In the same period the infection has been detected also in non-European countries such as Israel (Weisman et al., 1988), Yemen (Sarakbi, 1989), Japan (Uramoto et al., 1990), Mexico (Decanini et al., 1991), Morocco (Houadfi et al., 1991), Brasil (Arns and Hafez, 1992), Zimbabwe (Cadman et al., 1994), Taiwan (Lu et al., 1994), Caribe (Jones, 1996), Chile (Toro et al., 1998), Jordan (Gharaibeh and Algharaibeh, 2007), China and Nigeria (Owoade et al., 2008). The first detection of AMPV in North American has been

reported only in 1996 in Colorado (Senne et al., 1997), followed by an outbreak in Minesota in 1997.

The geographical distribution of the AMPV subtypes is interesting: meanwhile subtypes A and B showed a worldwide distribution, with the exception of Oceania and North America, the subtype C seems to be present only in the latest, even if more recently this subtype has been isolated in France and North Korea (Toquin et al., 2006; Lee et al., 2007). The subtype D has been detected only during an outbreak in France in 1985 and to date no others detection of this subtype has been reported (Bayon Auboyer et al., 2000).

2.1.2.3 Transmission

AMPV is a virus not able to resist out of the host. This evidence and the replication limited to the respiratory tract suggest that the direct contact transmission, both directly with infected animals or their respiratory discharges, is the most probable way of infection. It's highly unlikely the existence of vectors and the virus is not able to give latency in the host. Nevertheless, infected water, the movement of infected birds, equipment, personnel and feed trucks can play a role in the spread of the virus (Stuart, 1989). Some evidences suggest that a role in the transmission may be play by wild birds, but the transmission between wild and domestic birds has not been proven yet (Gough and Jones, 2008).

2.1.3 PATHOGENESIS

AMPV infected the upper respiratory tract: the nasal cavities, concha, infraorbital sinus and trachea are considerate not only the first replicative site of the virus, but

in general the main target tissues for viral replication. Less frequently the virus could be detected in the lungs and in the air sacs. In the tissues of the upper respiratory tract the virus can be detected by immunofluorescence up to 9 days post infection (d.p.i.) and can be isolated up to 14 d.p.i. in both turkeys and chickens, confirming that the tissues distribution and the replication rate are very similar in these two species (Catelli et al., 1998; Cook et al., 1991; Jones et al., 1988). Using molecular biology techniques, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), the viral RNA has been detected in the trachea up to 19 d.p.i. (Li et al., 1993). The virus can reach the reproductive apparel and replicates in the oviduct causing a reduction of the eggs production (Cook et al., 2000; Hess et al., 2004; Jones et al., 1988; Sugiyama et al., 2006; Villareal et al., 2007). Even if the mechanism of the spread to other organs is not clear, AMPV has also been occasionally detected in the Harderian gland, kidneys (Khehra and Jones, 1999), spleen, cecal tonsil and bursa of Fabricious (Aung et al., 2008). A transient viremia could be an explanation of the spread of the virus, but AMPV is rarely detected in the blood, thus this phenomenon needs to be investigating further (Shin et al., 2000b). The penetration to the lower tract of the respiratory apparel can be facilitated by bacterial co-infection. Several bacterial have proven to be involved in this process: *Escherichia coli* (Al-Ankari et al., 2001; Turpin et al., 2002; Van de Zande et al., 2001), *Bordetella avium* (Cook et al., 1991; Jirjis et al., 2004), *Mycoplasma gallisepticum* (Naylor et al., 1992) and *Mycoplasma imitans* (Ganapathy et al., 1998), *Riemerella anatipestifer* (Rubbenstroth et al., 2009), *Chlamydophila psittaci* (Van Loock et al., 2005) and *Ornithobacterium rhinotracheale* (Marien et al., 2005). The co-infection with bacterial can cause an exacerbation of the disease and can enhance the viral distribution in the host. Viral co-infections seem to have the opposite effect: Infectious bronchitis virus (IBV) inhibits AMPV replication in the upper respiratory tract. Considering the different

subtypes, no clear differences have been found in the pathogenesis (Aung et al., 2008; Shin et al., 2000b; Van de Zande et al., 1999).

2.1.4 SYMPTOMATOLOGY

The specie more severely affected is Turkeys, while in chickens the infection is often asymptomatic or with mild symptom. Affected turkeys showed the typical respiratory disease symptoms: coughing, sneezing, nasal discharge, swollen infraorbital sinus, conjunctivitis and submandibular oedema followed by depression and decrease in feed intake (Buys et al., 1989; Jones et al., 1986; McDougall and Cook, 1986). The morbidity is generally very high; it can reach 100%, while the mortality is highly variable, ranging from 0% to 50%, and age dependent (Hafez; 1993; Pattison, 1998; Stuart, 1989). The severity of the disease depends on management factors, such as birds' density, ventilation, temperature, hygienic conditions, and on secondary bacterial infections (Gough and Jones 2008; Hafez, 1993). Without any complications the recovery from the disease is quick and the symptoms generally disappear in 10-14 days (Cook, 2000a). In chickens the symptomatology is generally milder than turkeys, nevertheless the disease can be exacerbate by secondary bacterial infection. Co-infection with *E. coli* can lead to the Swollen Head Syndrome (SHS): not only respiratory signs characterize this disease, but it's also present a general head swelling, causing neurological signs, such as disorientation, torticollis and opistothonus (Hafez, 1993; Jones et al., 1991). In both the species, AMPV infection can cause drop in egg production. In turkeys the drops in eggs production can reach even 70%, but generally it assets between 10% and 20% (Schiricke, 1984; Wyeth, 1990). Associated with the drop of the eggs production, it's usually observed a decrease in the quality of the eggs shell (Drouin et al., 1985). Drops in egg production have been reported in field in laying hens too,

ranging from 2% to 40% in association with poor egg quality (Drouin et al., 1985; O'Brian, 1985; Picault, 1988). Nevertheless in experimental conditions only the injection of virus is able to decrease the laying performance in chickens (Cook et al., 2000; Hess et al., 2004; Sugiyama et al., 2006).

Figura 2.3 Swollen head syndrome in chicken

2.1.5 POST-MORTEM

2.1.5.1 Gross lesions

In turkey has been observed an inflammatory case of the first respiratory tract including the presence of watery to mucoid exudate. It has also been observed welling of the infraorbital sinus caused by accumulation of mucus, conjunctivitis and submandibular oedema (Stuart, 1989). In breeders, along with the lesion of the respiratory tract, may be seen prolapsed oviducts, folded shell membrane in the reproductive tract and egg peritonitis (Jones et al., 1988; Jones et al., 1991). In case of bacterial co-infections the clinical case can be more severe and airsacculitis, pericarditis, perihepatitis and pneumonia may be seen (Stuart, 1989). In chickens the lesions tend to be similar, but milder in the absence of bacterial co-infection (Catelli et al., 1998). *E. coli* co-infection can lead to rhinitis and sinusitis mucoid purulent exudate, infraorbital oedema, airsacculitis, pericarditis and accumulation

of a yellow gelatinous or even purulent oedema in the subcutaneous tissues of head and neck (Al-Ankari et al., 2001; Picault et al., 1987).

2.1.5.2 Microscopic lesions

The main histological lesions observed during AMPV infections are borne to the cells of the respiratory epithelium and no differences have been observed between turkeys and chickens. In the epithelial cells can be observed eciliation, deepithelization, and thickening of the mucosa, hyperaemia, mononuclear infiltration and glandular proliferation in the turbinates, infraorbital sinuses and trachea. The process of recovery of the epithelial tissue begins 14 d.p.i., while the fully recovery is reach between 18 and 21 d.p.i. (Aung et al., 2008; Catelli et al., 1998). In the Swollen head syndrome, a part from the lesions listed above, have been reported periostitis, otitis and meningitis (Hafez, 1993). Epithelial damage of the oviduct has also been seen in both the species (Cook el al., 2000).

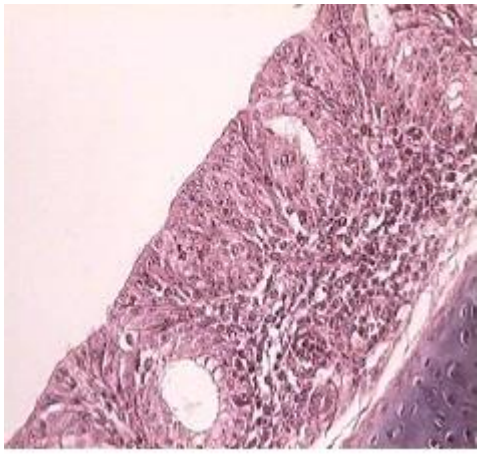


Figure 2.4. Microscopic lesions in the trachea due to AMPV infection

2.1.6 IMMUNE RESPONSE

As most of the respiratory pathologies, local immunity plays a central role in the prevention of the infection: in experimental condition has been observed a lymphocyte proliferation of the first respiratory tract and in the Harden's gland, resulting in an increase levels of IgA and IgG in the tears and in the trachea (Cha et al., 2006; Liman and Rautenschlein, 2007; Rautenschlein et al., 2011; Sharma et al., 2002;). However, the local immunity response doesn't last long, explaining recurrent infections during bird's productive life in farms (Rautenschlein et al., 2011). Considering the systemic immunity, the cellular mediate immunity is also critical for the response to the infection, while the humoral response seems not to be. As suggested by experimental studies and field evidences, circulating antibody titres do not seem to be an indicator of protection: turkeys with no detectable antibody titres resulted to be protected against a virulent strain challenge (Cook et al., 1989; Rautenschlein et al., 2011). Maternal immunity cannot prevent the infection, however chicks with high level of maternal antibodies showed milder symptoms compare to chicks without any maternal antibodies (Naylor et al., 1997a). More important, maternal immunity does not affect early vaccination, allowing young chicks to be immunized in early stages or directly *in ovo* (Cook et al., 1989; Worthington et al., 2003). It has been proven the interference of other viruses in the immunity response against AMPV: in particular the co-vaccination with vaccines against IBV or Newcastle disease virus (NDV) cause a significance decrease of AMPV antibody titre (Ganaphaty et al., 2006; Jones et al., 1998).

2.1.7 DIAGNOSIS

AMPV clinical signs and post-mortem findings are too much similar to those of other respiratory pathogens, both viral and bacterial, not allowing a differential diagnosis. Thus the viral identification is crucial for the diagnosis. Isolating the virus, detecting the viral genome or proteins or demonstrating the specific serological response of the host could achieve the viral identification (Gough and Petersen, 2008).

2.1.7.1 Virus isolation

Virus isolation could be a difficult task, due to the short persistence of the virus in the host (Catelli et al., 1998; Cook et al., 1991). The time of the sampling, between 3 and 5 d.p.i. from birds not yet showing any clinical signs, together with the conservation of the samples, are crucial factors for the virus isolation (Cook and Cavanagh, 2002). The primary isolation of AMPV could be done in 6 days old embryonated specific pathogens free (SPF) eggs via yolk inoculation or in tracheal organ colture (TOC) (Buys et al., 1989; McDougall and Cook, 1986; Panigrahy et al., 2000; Wyeth el al., 1986). After several passages the presence of embryonic haemorrhages, or in last instance the death of the embryo, are signs of positive isolation of the virus (Buys et al., 1989; Cook et al., 1999). After inoculation in TOC the presence of the virus is demonstrated by the ciliostatic effect between 3 and 5 days post inoculation (Cook et al., 1991). TOC seems to be the most convenient isolation method, having a better sensitivity and being faster and cheaper compare to embryonate eggs isolation (Naylor and Jones 1993). However, this method is not suitable for subtype C isolation, because this subtype is not ciliostatic (Cook et al., 1999). Once isolated, the virus could be adapted to several cell lines, such as VERO

cell, chick embryo fibroblast and chick embryo liver cell monolayer (Buys et al., 1989; Grant et al., 1987; Williams et al., 1991). However, the embryo haemorrhages, the ciliostatic effect and the cytopathic effect (CPE) are specific but not exclusive of AMPV, therefore the identification of the virus needs to be investigate further using other methodologies, such as immunofluorescence (Jones et al., 1988), immunoperoxidase (Catelli et al., 1998), RT-PCR (Dani et al., 1999; Juhasz and Easton, 1994; Naylor et al., 1997a).

2.1.7.2 Viral detection

A faster and easier approach for the detection of the virus is the used of immunohistochemistry techniques such as Immunofluorescence (IF) and Immunoperoxidase (IP)(Catelli et al., 1998; Jones et al., 1988). The used of monoclonal antibodies allowed the differentiation between the different subtypes (Collins et al., 1993; Cook et al., 2003). However, these techniques are now a day overcome by molecular biology techniques, such as RT-PCR. RT-PCR, amplifying a region of the viral genome, showed to be very suitable for diagnosis, being fast, sensitive and reproducible (Gough and Jones, 2008). Moreover, due to its high sensitivity, the RT-PCR is able to detect virus for a longer period compare to the techniques mentioned before (Cook and Cavanagh, 2002). Several RT-PCRs have been developed, targeting different AMPV genes, showing different sensitivity and subtype specificity: a protocol targeting the highly conserved region of the N gene, has shown to be able to detect all the four subtypes, but not to differentiate between them (Bayon-Auboyer et al., 1999). RT-PCR targeting the F and the G gene enabled the differentiation of subtypes A and B (Jing et al., 1993; Mase et al., 1996; Naylor et al., 1997b). Similar protocols were developed to differentiate subtype C from the other subtypes (Ali and Reynolds, 1999; Pedersen et al., 2000). Furthermore, has

been developed a multiplex RT-PCR protocol able to detect AMPV and other respiratory RNA viruses as avian influenza (AI), IBV and NDV (Ali and Reybolds, 2000; Gorashi et al., 2007; Malik et al., 2004). Recently has been developed a RT-PCR protocol, followed by endonuclease restriction analysis, able to differentiate between vaccine and field strains (Listorti et al., 2014). The advent of the Real Time PCR allowed a further step forward in AMPV detection: in recent years Real Time RT-PCR protocols able not only to detect and differentiate the virus with a better sensitivity, but also to obtain a viral quantification have been developed (Cecchianto et al., 2013; Cecchinato et al., 2014; Guioine et al., 2007). The virus could be also identified using serological tests, such as virus neutralization (VI), enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF). Antibodies against AMPV persist in the sera up to 90 d.p.i., therefore these tests are very suitable to confirm the infection in field studies (Gough and Jones, 2008; Jones et al., 1988). Especially ELISA tests have shown to be very suitable for mass serological test. The lack of the ELISA test is that the efficiency is related to the coated antigen: this mean that homologous tests have shown good performance while heterologous test shown poor performance, leading even to false negative, as observed using A and B type ELISA to detected antibodies against the C type (Cook et al., 1999; Cook and Cavanagh, 2002; Maherchandani et al., 2005; Mekkes and de Witt, 1998; Toquin et al., 1996). To avoid the subtype specificity of the ELISA test, in recent years blocking ELISA protocols have been developed (Catelli et al., 2001; Turpin et al., 2003).

2.1.8 DISEASE CONTROL

A specific therapy against AMPV, is not available, therefore is important to actuate a preventive approach in order to avoid the spread of the disease. Vaccination is the

key point of the preventive approach, in fact several kind of vaccines are available and largely used in commercial poultry. Live attenuated vaccines are generally administrated in the early stage of life of different categories of commercial poultry, by intranasal inoculation, eye-drop, spray or drinking water (Cook, 2000b; Gough and Jones, 2008). Dependently on the bird category, more than one vaccination is needed: e.g. for broiler the vaccination at early stages is generally enough to fully protect the birds thru their entire productive life, while in growing turkeys more vaccination are needed. The situation changes for laying bird; those animals require a vaccination using an inactivated vaccine just prior the onset of the lay, in order to avoid a decrease in eggs production (Cook et al., 1996a; Cook, 2000b). Cross protection between different subtypes has been observed; subtype A vaccine confer protection against the B type and the other way round. Nevertheless, subtype C vaccine cannot protect against subtype A and subtype B (Cook et al., 1995; Cook et al., 1999; Eteradossi et al., 1995; Toquin et al., 1996). The co-vaccination with live vaccines against respiratory viruses, such as IBV and NDV, has been shown to cause a decrease in AMPV vaccine replication (Cook et al., 2001; Ganapathy et al., 2005; Ganapathy et al., 2006). In order to avoid this interference two routes have been followed: the first one involved the vaccination in ovo against IBV and the other one the development of new generation vaccines, such as recombinant vaccines and subunit vaccines (Falchieri et al., 2013; Hess et al., 2004; Hu et al., 2011; Kapczynski and Sellers, 2003; Qingzhong et al., 1994; Tarpey et al., 2001; Tarpey and Huggins, 2007; Worthington et al., 2003).

2.2 REVERSE GENETICS LITERATURE REVIEW

The development of reverse genetic (RG) techniques for non-segmented negative stranded (NNS) RNA viruses has been a big step forward in viral research. Generation of viruses derived from DNA copies (cDNA) of their genome has allowed scientists to study the effect of specific mutations on viral biology and to perform major sequence changes, such as deletion or genes addition (Conzelmann and Meyers, 1999; Conzelmann, 2003; Walpita et al., 2005). Since RG has been established, several NNS RNA recombinant viruses expressing exogenous genes have been generated to develop improved or multivalent vaccines (Neumann et al., 2002; Sato et al., 2011).

These viruses have been shown to be suitable candidate as vectors form several reason: integration of the foreign gene into the host genome is very unlikely, because NNS RNA virus do not replicate through DNA intermediates; recombination is an extremely rare event; the genome organization is quite simple, generally 5-11 proteins and genes, making manipulations easier; they grow to high titres and express high levels of proteins; they are able to induce strong humoral and cellular immune responses (Conzelmann and Meyers, 1999; Walpita et al., 2005); studies proved they’re able to accept and express foreign genes without mutations incurring over several passages (Mebastion et al., 1996; Schnell et al., 1996).

A reverse genetics system for AMPV subtype A was developed for the first time in 2004 by Naylor et al.. A full length (FL) cDNA of subtype A was cloned in a plasmid vector including a kanamicyn- resistant gene, essential in the cloning process, a T7 promoter and Hepatitis delta virus ribozyme (HDRV) (Naylor et al., 2004). Similarly, the genes coding for the support proteins essential to form the ribonuclear complex (RNP), N, P, L and Matrix 2 (M2) were also cloned in other

plasmids lead by a T7 promoter (Naylor et al., 2004). VERO cell infected with a recombinant Fowlpox virus expressing the bacteriophage T7 polymerase were used as substrate for the viral rescue. The T7 polymerase expressed by the recombinant Fowlpox virus is able to recognize the T7 promoter inserted in the plasmids and then to initiate transcription directly from them, allowing the formation of the RNP (Naylor et al., 2004). After the complex has been established, genome replication and gene transcription can begin as occur naturally, producing new RNA virions. Two years later, a reverse genetics system was similarly developed in the USA for suntype C, but to date, the attempt to develop a reverse genetics system for subtype B failed (Govindarajan et al., 2006). The development of these systems allowed investigating further the behaviour of the virus, thru the insertion of multiple mutations (Brown et al., 2011; Naylor et al., 2007; Naylor et a., 2010) and genes deletion (Govindarajan et al., 2010; Ling et al., 2008; Whelan et al., 2004). More important, the AMPV reverse genetics systems have shown that the virus is able to accept and express exogenous genes, electing AMPV as vector for the development of recombinant live vaccines (Govinfarajan et al., 2006; Lupini et al., 2008). In 2013 Falchieri et al., demonstrated that AMPV-A is able to accept and express infectious bronchitis virus (IBV) exogenous genes and to induce a partial protection at the challenge.

3. GENERAL MATERIALS AND METHODS

In this chapter are described the general materials and methods used throughout the studies. Any deviations from the materials and methods described below will be specified in the following chapter.

3.1 Nucleic acid extraction

3.1.1 Viral RNA was extracted and purified using Qiamp viral RNA mini kit (Qiagene), following the manufacturer recommendation.

3.1.2 Plasmid DNA was extracted and purified using Qiamp viral miniprep mini kit (Qiagene), following the manufacturer recommendation.

3.2 Reverse transcription

Reverse transcription of genomic viral RNA or mRNA was performed using Super Script^{TM} III Reverse Transcriptase (Invitrogene). The enzyme was always added at the reaction mix at 50°C in order to avoid mispriming.

RT reaction mix	
Reagent	**Quantity**
5 x First-Strand Buffer	4µl
DTT (0.1M)	2µl
dNTP solution (40mM)	1µl
primer (10µM)	1µl
Extracted RNA	2µl
Rnasin (Promega)	0.5µl
Water (Invitrogen)	up to 20µl
Mineral oil (Sigma)	50µl
Super Script^{TM} III (200u/ µl)	1µl

Table 3.1 Reaction mix used to reverse transcribed the viral RNA.

RT cycle	
Temperature	**Duration**
70°C	1 minute
50°c	2 minutes
50°c	Hot Start
50°C	90 minutes
94°C	10 minutes
12°C	Hold

Table 3.2 Cycle used to transcribed the viral RNA.

3.3 Polymerase chain reaction

In the studies were used different polymerases depending upon the required amplicon size and the following applications. The PCR products to be used in site directed mutagenesis (SDM) and ligation were generated using PfuTurbo DNA polymerase (Agilent Technologies). The short screening PCRs and sequencing PCRs were performed using GoTaq Flexi DNA polymerase (Promega). To avoid mispriming, Pfu Turbo was added to the reactions mix at 80ºC.

Pfu Turbo reaction mix	
Reagents	**Quantity**
10X Buffer	5µl
dNTP solution (40mM)	1µl
Forward primer (10µlM)	1µl
Reverse primer (10µM)	1µl
Template	1µl to 5µl
Pfu Turbo DNA Polymerase (2.5u/µl)	1µl
Water (Invitrogen)	Up to 50µl
Mineral oil (Sigma)	50µl

Table 3.3 Reaction mix used to amplify DNA or cDNA to be used in SDMs or ligations.

Pfu Turbo cycle		
Temperature	**Duration**	**Number of cycle**
80°C	10 seconds	1
80°C	Hot start	1
94°C	5 seconds	5
50°C	20 seconds	
68°C	60 seconds per kb	
94°C	5 seconds	25
50°C	20 seconds	
68°C	60 seconds per kb with 10 seconds time incremented	
12°C	Hold	

Table 3.4 Amplification cycle used to amplify DNA or cDNA to be used in SDMs of ligations.

GoTaq reaction mix	
Reagents	**Quantity**
5X Go Taq Flexi Buffer	10µl
MgCl2 solution (25mM)	3.5µl
dNTP solution (40mM)	1µl
Forward primer (10µlM)	1µl
Reverse primer (10µM)	1µl
Template	2µl
GoTaq DNA Polymerase (5U/µl)	0.25µl
Water (Invitrogen)	Up to 50µl
Mineral oil (Sigma)	50µl

Table 3.5 Reaction mix used for the short screening PCR and sequencing.

GoTaq cycle		
Temperature	**Duration**	**Number of cycle**
94°C	15 seconds	1
94°C	10 seconds	35
50°C	20 seconds	
68°C	40 seconds	
12°C	Hold	

Table 3.6 Cycle used for the short screening PCR.

3.4 Agarose gel electrophoresis

To visualise the PCR products, the SDM products, to check the integrity of the plasmids and to quantify them, was used agarose gel electrophoresis. Depending on the size of the bands to be visualised, the gel were prepared with concentrations ranging from 0.8% to 2% w/v using TBE buffer (Invitrogen) diluted 10 times added with Red Safe Nucleic Acid staining solution (Intron Biotechnology) to visualized the bands under U.V. light. 5 to 10µl of each sample, depending on the nature, were mixed with 5µl of loading buffer and then loaded onto the gel. In each run molecular weight markers HyperLadder I (Bioline) was included: this weight markers enable DNA quantification.

3.5 Sequencing

Before the sequencing all the PCR products were purified using Shrimp Alkaline Phosphatase (SAP) (Usb) and Exonuclease I (EXO) (Usb) to dephosphorylate and degrade residual dNTPs and primers. The purified amplicons were submitted to Source Bioscience Sequencing (Cambridge UK). The sequences were then visualised using Chromas, aligned and analysed using both Bioedit Sequence Alignament Editor and Generunner.

3.6 Ligations

XhoI and SalI are two restriction enzymes (RE), which recognise to different site, although they both create the same 5' overhang. After digestion the two different sites can be ligated. This leads to a sequence that does not contain the complete recognition sequences of either RE and therefore cannot be digested by them. These features have been used in these studies to circularised plasmids and to ligate PCR products into plasmids. All the ligations, were performed using T4 DNA ligase (Fermentas) in the presence of the XhoI (Invitrogen) and SalI (Invitrogen): the ligation mixtures were incubated at 14°C for at minimum 2 hour.

Ligation reaction mix	
Reagents	**Quantity**
5X Ligation Buffer	2µl
XhoI (10u/µl)	0.5µl
SalI (15u/µl)	0.5µl
Plasmid	0.5µl to 2µl
Amplicon	1µl to 5µl
T4 DNA ligase (30u/µl)	0.5µl
Water (Invitrogen)	Up to 20µl

Table 3.7 Reaction mix used to cicularised plasmids and to ligate PCR products into plasmids.

3.7 Site directed mutagenesis

Site directed mutagenesis (SDM) was performed both using primer pairs and blunt-end PCR products, also called megaprimers. The technique has been used to introduce useful point mutations, multiple nucleotide substitution or exogenous genes. All SDMs were performed using PfuTurbo DNA polymerase (Agilent Technologies) as enzyme.

Pfu Turbo reaction mix	
Reagents	**Quantity**
10X Buffer	5µl
dNTP solution (40mM)	1µl
Forward primer (10µlM)	1µl
Reverse primer (10µM)	1µl
Plasmid template	1µl to 5µl
Pfu Turbo DNA Polymerase (2.5u/µl)	1µl
Water (Invitrogen)	Up to 50µl
Mineral oil (Sigma)	50µl

Table 3.8 Reaction mix used in SDM reactions.

If megaprimers were used in the SDM, a 5µl volume of the amplicons was added to the reaction mix.

Pfu Turbo cycle		
Temperature	**Duration**	**Number of cycle**
80°C	10 seconds	1
80°C	Hot start	1
94°C	30 seconds	1
94°C	30 seconds	18
50°C	60 seconds	
68°C	30 minutes	
12°C	Hold	

Table 3.9 Cycle used in SDM reactions.

Each SDM product was treated with DpnI (Agilent) enzyme in order to remove the original methylated plasmids. 10µl of the SDMs were incubated at 37°C for at minimum 2 hours with 1µl of DpnI (10u/µl), followed by a step at 60°C for 20 minutes to inactivate the enzyme.

3.8 Transformation and liquid culture

All transformations were carried on using Max Efficiency STB12 Competent Cells (Invitrogene). The transformation protocol adopted is the following: 1µl of SDMs or ligations products was gently added to about 100µl of STB12 cells and incubated for 30 minutes in ice, then heat shocked at 42°C in water bath for 25 seconds and eventually for further 2 minutes in ice. 250µl of SOC medium was then added to the transformation mixture and the samples were agitated and incubated at 25°C for 90 minutes. After the incubation the whole volume of each transformation mixture was inoculated onto LB agar plates, added with Kanamycin antibiotic at a concentration of 15µg/ml and incubated for 24 to 72 hours at 25°C. Positively transformed cells carried the plasmid Kanamycin resistance gene, allowing them to grow in the presence of that antibiotic. Colonies were screened by PCR and the positives were liquid cultured in 15ml LB broth (Gibco) containing Kanamycin at a concentration of 15µg/ml. Liquid cultures were agitated and incubated at 25°C for 24 up to 72 hours.

3.9 Restriction enzyme digestion

Restriction enzymes were used in these studies for two different purposes: 1- plasmid DNA mapping and quantification, 2- preparation of PCR products and plasmids DNA for sticky ends ligation. For the first purpose were used EcoRI

(Invitrogen), for the second one XhoI and SalI. All restriction enzyme mixtures were incubated 2 hours at 37°C, following manufacture recommendation.

4. A COMPARISON OF AMPV SUBTYPE A AND B FULL GENOMES, GENE TRANSCRIPTS AND PROTEINS LED TO REVERSE GENETICS SYSTEMS RESCUING BOTH SUBTYPES.

4.1 INTRODUCTION

Avian rhinotracheitis is a major disease affecting domestic poultry throughout most of the world and is caused by infection with avian metapneumovirus (AMPV). Four AMPV subtypes (A to D) have been discovered and of these subtypes A and B are considered responsible for most AMPV related disease in chickens and turkeys outside of the USA. The extensive use of live vaccines of both A and B subtypes has made it difficult to accurately assess the relative prevalence of each subtype in the field in many world regions, but nonetheless subtype B field strains are generally accepted to be dominant in Western Europe, and for this reason, vaccination with this subtype has been prioritised (Cecchinato et al., 2014).

For more than ten years, the availability of subtype A reverse genetics (RG) systems (Ling et al., 2008; Naylor et al., 2004) has allowed subtype A virus genomes to be modified and the resultant phenotypes investigated. Within suitable cells, full length DNA viral copies, transcribed to RNA in the presence of a number of essential AMPV proteins, produce the remaining viral proteins, then viruses with sequences matching the genome copy. Using this RG tool, effects of some precise genetic changes on virus properties have been determined, in terms of gene deletions (Ling et al., 2008; Naylor et al., 2004), virulence (Brown et al., 2011), protective capacity of live vaccines (Naylor et al., 2010) and gene insertions (Falchieri et al., 2013).

Generally in mononegavirales reverse genetics systems, the viral polymerase replicates N protein encapsidated RNA antigenome in association with the P protein, and for the family Pneumovirus transcription factor M2 protein, as has been reviewed previously for similar viruses (Whelan et al., 2004). Specific genome sequences are known to be involved in regulation of polymerase attachment, genome replication, transcription initiation, transcription termination and the balance of genome and antigenome copies, but for AMPV most details of these sequences remain unknown. For genome replication, the viral polymerase must recognise replication signals but ignore transcription start/stop signals, whereas for transcription, these signals must be recognised.

Comparison of complete genome sequences has shown that subgroups A, B and D are more related to each other than subtype C (Brown et al., 2014) and another comparison of subtypes A, B and C showed subtype A and B to have the most similar genomes (Jacobs et al., 2003). Subtypes A and B also appear to be most similar in their species specificity and behaviours in the field, hence live subtype A and B vaccines have been employed largely interchangeably to control disease in commercial turkeys and chickens, albeit with an increasing bias toward subtype B. Cross protection and antigenic studies have suggested that some protective and antigenic differences do exist (Collins et al., 1993; Cook et al., 1993; Van de Zande et al., 2000) and this highlighted the need for a reverse genetics system to enable the generation of improved live subtype B vaccines, as well as to understand other properties of this subtype.

A project to develop a subtype B reverse genetics system was initiated in our laboratory soon after the subtype A development (Naylor et al., 2004) but encountered problems. Also at a similar time other groups were known to have

initiated similar ventures yet no system was forthcoming. In our case this was due to problems encountered while attempting to clone larger subtype B genome sections into the plasmids previously found successful for cloning subtype A viruses. While N, P and M2 genes could be readily cloned, the L gene and full genome proved impossible, as sequences proved toxic even using the specialist tolerant cloning bacteria previously found adequate for subtype A. This either led to the complete absence of clones, or clones containing major deletions, often of several thousand nucleotides.

With a view to potentially utilising some of the available subtype A RG system components in the development of a subtype B system, it was decided to investigate properties of subtype A and B viruses likely to affect rescue and replication. Leader and trailer sequences essential for attachment of the viral polymerase were determined and compared, as were those sequences recognised by the viral polymerase in initiating and terminating the transcription of individual viral genes. The study further compared protein similarities, especially for N, P, M2 and L which are all directly involved in encapsidation, replication and transcription of the genome in a reverse genetics system. In most cases we report for the first time the individual gene transcription stop signals for both subtype A and B virus genes, as well as many previously unreported leader and trailer sequences. While many gene stop sequences were predictable from available genome sequences, others were not, especially where more than one termination like sequence was present at a gene end, as for example seen with the M2 and G genes. When combined, results of these studies suggested that subtype A and B reverse genetics systems might be able to recover full genome copies of the opposite subtype. Due to the importance of AMPV subtype C in North America and elsewhere, comparison included an established virus from that subtype.

During the investigation cloning attempts were continued and during these, a literature search brought to our awareness a commercial plasmid pSMART that had permitted problematic regions of an influenza virus genome to be successfully cloned (Zhou et al., 2011). This was applied in cloning the subtype B full genome and L gene. Finally a subtype B cloned genome was rescued with either subtype A or B support components, hence this study includes report of the first AMPV subtype B reverse genetics system. We also demonstrated the rescue of a subtype A virus using this subtype B reverse genetics system.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

The subtype A (Germany A) virus used to create the first AMPV reverse genetics system was isolated in Germany in the 1990's (Naylor *et al.*, 2004) and was later tested in vaccination studies (Naylor *et al.*, 2010). Other subtype A field viruses sequenced for gene sequence comparison were #8544(Jones *et al.*, 1986), Italy 259 (Cecchinato *et al.*, 2010), UK 3B (Mcdougall & Cook, 1986), CVL 14-1 (Collins & Gough, 1988) and UK CP/1 (Jones *et al.*, 1991); and commercial live vaccines Poulvac TRT (Fort Dodge), Nobilis TRT (Intervet) and Turkadin (discontinued).

The subtype B virus used to create the first AMPV subtype B reverse genetics system was a vaccine strain derived from UK strain 11/94. Subtype B field viruses sequenced for gene sequence comparison were Italy 205 and 240 (Cecchinato *et al.*, 2010), France 147 and 38 (Cook *et al.*, 1993), Netherlands 27 (Cook *et al.*, 1993), Italy 16-91(Cook *et al.*, 1993); and commercial live vaccines Nemovac (Merial), Aviffa (Merial) and Nobilis Rhino CV (Intervet).

4.2.2 Determination of leader and trailer sequences

RNA of subtype A was extracted from from #8544, Poulvac TRT, Italy 240, RhinoCV, Nemovac and from some recombinant rescued viruses using QIAamp Viral RNA mini kit (Qiagen, France, Courtaboeuf) according to the manufacturer's instructions (3.1).

Leader and trailer were determined by 3'RACE on the genome and antigenome respectively following the protocol described by Brown et al. (2013). Briefly: the viral negative sense RNA genome and positive sense replication intermediate were poly A tailed by incubation with E-PAP Poly(A) tailing polymerase (Ambion Invitrogen France, Illkirch) at 37 degrees for 1hour. The poly A tailed RNAs were purified using NucAway spin columns (Ambion: Invitrogen France, Illkirch) according to manufacturer's recommendations. Reverse transcription (3.2) was performed using a mixture of 3 primers each starting with an adaptor sequence of 19 base pair at the 5′ end followed by 21 bases complementary to the poly A tail and finally an anchor base at the 3′ end (the primers differ only in the bases used as anchor). The ends of the cDNAs of the genome and the positive sense replication intermediate were amplified by PCR (3.3), using a primer of the same sense as the adaptor sequence and one subtype specific reverse primer for the 3′ end and a subtype specific forward primer for the 5′ end. The amplicons were sequenced and the analyses (3.5). Primers listed in table 4.1.

4.2.3 Determination of 3' termini sequence of subtype A and B AMPV mRNAs

RNA of the viruses listed in the previuos chapter was extracted using QIAamp Viral RNA mini kit (Qiagen, France, Courtaboeuf) according to the manufacturer's

instructions (3.1). The mRNA was amplified by RT-PCR using a method described by Brown et al. (2011). The mRNA was reverse transcribed (3.2) using a mixture of 3 primers each starting with an adaptor sequence of 19 base pair at the 5′ end followed by 21 bases complementary to the poly A tail and finally an anchor base at the 3′ end (the primers differ only in the bases used as anchor). The cDNAs were then amplified by PCR (3.3) using a gene specific forward primer and a primer matching the adaptor. The PCR products were sequenced toward the polyA tail using the same gene specific primers. The sequences obtained were aligned and analysed (3.5). Primers listed in table 4.1.

4.2.4 Determination of viral gene sequences and their comparison

Sequences of subtype A and B virus genes were as determined by sequencing of PCR amplified genome sections, as described in previous studies (Brown *et al.*, 2011; Cecchinato *et al.*, 2010; Naylor *et al.*, 2004; Naylor *et al.*, 2007). Using Bioedit, nucleotide sequences aligned and inter-subtype identities calculated, then sequences were translated to allow predicted amino acid identities and similarities to be calculated.

4.2.5 Construction of subtype B reverse genetics system

4.2.5.1 Preparation of pSMART plasmid vector

pSMART vector was used to clone the AMPV-B FL genome copy. Prior the cloning, the vector have been phosphorylated, using T4 polynucleotide kinase (Promega), following the manufacturer recommendation, ligated (3.6) and

transformed on E. coli competent cells (3.9). An XhoI site was eventually introduced by SDM (3.7) to allow the ligation of the AMPV-B amplicons.

4.2.5.2 Subtype B genome copy construction

To generate the avian matapneumovirus (AMPV) subtype B DNA full genome copy was adopted a strategy based on a series of RT-PCR, SDM and ligation steps (Figure 4.1).

RNA was extracted from a RhinoCV (Intervet) vaccine (3.1). Two overlapping cDNA sequences were obtained by reverse transcription (3.2) and the cDNA was used as template for three PCRs (3.3) in order to cover the AMPV-B genome sequence from the leader to position 12.0kb (3.3). The amplicons, 0-4kb, 4kb-8kb, 8kb-12kb, were generated using primers listed in table 4.2 that introduce SalI site at each end of the products. The primers to generate the leader of the genome contain a T7 promoter sequence at the 5' end. The last section of the genome was copied by high fidelity PCR (3.3) from a plasmid containing the AMPV-B sequence from position 12.0kb to 13.5kb and the sequence of the Hepatitis Delta Virus Ribozyme (HDVR).

8kb-12kb amplicon was ligated into the modified pSMART plasmid (3.6). The plasmids were transformed (3.8) and the colonies screened by PCR (3.3) using primers chosen at either side of the junction. Colonies positive at the screening were cultured on LB Broth (3.8), the plasmids purified (3.1) and checked for integrity by restriction endonuclease (RE) analysis (3.9). The generated plasmids underwent SDM (3.7) to introduce an XhoI site at the 12kb end. The XhoI site was used to ligate the 12.1kb-13.5kb product (3.7). Following the same protocol the 4-kb-8kb and then the 0-4kb amplicons were cloned into the plasmids, as showed in figure

4.1. The full-length (FL) plasmids generated were sequenced (3.5). The sequences obtain were aligned against RhinoCV one (3.5).

4.2.5.3 Preparation of B type support plasmids

N, P and M2 sequences were amplified by RT-PCR from RhinoCV vaccine. The RNA was extracted (3.1), reverse transcribed (3.2) and amplified using high fidelity polymerase (3.3). Amplicons were cloned into the same plasmids as had been used previously in the subtype A rescue system (Naylor *et al.*, 2004). For the L gene, because of cloning stability issues with the original plasmid used to clone the subtype A L, it was copied by hi-fidelity PCR (3.3) from the cloned full subtype B genome to include the pSMART LC Kan sequence. This was ligated (3.6) and cloned (3.8). The plasmids generated were cut with RE to check for integrity (3.9) and sequenced (3.5).

RT primers		
Primer	**Sequence**	
Dta-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTA	
Dtc-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTC	
Dtg-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTG	
Adaptor		
Adaptneg	GCATCTCGAGGCTTGTGGCT	
PCR and sequencing primers subtype A		
Gene	**Primer**	**Sequence**
Leader	N 2-	GCATGCCTACCTCTGCTG
N	N 1+	CAATATAATGTTGGGCCATG
P	P 1+	GCAATGATAGGGATGAGA
M	M 7+	GAAGCCATATGGTATGGTCTC
F	F 3+	GTGTGAGTTGCTCCATTGG
M2	M2:4+	GTCTCCCAGAGAAAAACT
SH	SH 2+	GCAACTAAGTGCTGCTAC
G	G 7+	GAAAAGACATTCAGTACATAC
L	L 10+	GGGAGTAAACTATCAGGATCGG
Trailer	L 19+A	GAAGTGGTTAAATCACGTTCTG
PCR and sequencing primers subtype B		
Gene	**Primer**	**Sequence**
Leader	N 2-	GCATGCCTACCTCTGCTG
N	NAB 1+	TCAAATACCCAAGAACCAAAAGCCGTC
P	PAB 1+	CCGACCCTGACGAAGATAATGATG
M	B 2.28+	CTGCTGGACCAGCTAAAAACTC
F	FAB 2+	ATGACTATGTGTTCTGTGATACTGCAGC
M2	M2AB 1+	GAATCCAGCAAATCTCATAAACAGTCTCAAG
SH	SHAB 1+	CAGAGCTGAGCACAACTACAGC
G	G15+B	GCAAGACGACCGACCAGAGAC
L	LAB 12+	CACAGCTCCTTGCTATGGAGAGG
Trailer	B 13.15+	CAAACCTAACACACTTGGACAACTCC

Table 4.1 Primers used to determine leader, trailer and transcriprion stop sequences

Primer		Sequence
pSMART Xho +		CCTGAATGATATCAAGCTTGAATTCCTCGAGGAATTCTCTAGATATCGCTCAATACTG
pSMART Xho -		CAGTATTGAGCGATATCTAGAGAATTCCTCGAGGAATTCAAGCTTGATATCATTCAGG
RT Primer		
Fragments	**Primer**	**Sequence**
0-8kb	B 8.38 neg	GAGCACTCTTCCTGTTTTCTCCAACAAAC
8kb-12.0kb	LAB 1+	CTGGAAGTGTCACAGACCAGTGC
PCR Primer		
Fragments	**Primer**	**Sequence**
0kb-4kb	APV lead T7 Sal+	GTCGACTAATACGACTCACTATAGGGACGAGAAAAAAAACGC
	B 4.0 Sal-	TAAGTCGACGTTGATATGTTTTGGTTGC
4kb-8kb	B 4.0 Sal+	CATATCAACGTCGACTTACCCTTGCAAAG
	B 8.08 Sal-	AAACTCGTTGGTCGACTCCTAAATCG
8kb-12.1kb	B 8.08 Sal+	AAACTCGTTGGTCGACTCCTAAATCG
	AVIF 12.1 Sal-	GTCATAGCATGTCGACTGTCTGAGTAAC
12.1kb-13-5kb	AVIF 12.1 Sal+	GTTACTCAGACAGTCGACATGCTATGAC
	CTPE 110 Sal+	CTTCCCCGTCGACGATGTCGGCG
SDM Primes		
Position	**Primer**	**Sequence**
Psmart cloning site	Psmart 220 Xho+	CGTCTTGCTCAAGGCCGCGATTAAATT
	Psmart 220 Xho-	AATTTAATCGCGGCCTTGAGCAAGACG
4.0kb	B 4.0 Xho+	GCAGTGCAACTCGAGCATATCAAC
	B 4.0 Xho-	GTTGATATGCYCGAGTTGCACTGC
8.0kb	B 8.08 Xho+	CTAGGACTCGAGAGCAAACTCGTT
	B 8.08 Xho-	AACGAGTTTGCTCTCGAGTCCTAG
12.1kb	AVIF 12.1 Xho+	GTTACTCAGACACTCGAGATGCTATGAC
	AVIF 12.1 Xho-	GTCATAGCATCTCGAGTGTCTGAGTAAC
11542bp	B 11542 +	GAAGCAACTCAAATGCAGAGAGAATTGCAACTGAG
	B 11542 -	CTCAGTTGCAATTCTCTCTGCATTTGAGTTGCTTC

Table 4.2 Primers used to generate subtype B full genome copy

RT Primer		
Gene	**Primer**	**Sequence**
N and P	Ac-Le-Trail +	ACGAGAAAAAAACGC
M2	FAB 1+	GCTAAAACAATAAGATTAGAAGGGGAGGTG
PCR Primer		
Gene	**Primer**	**Sequence**
N	N Start + B	GTCTCTTGAAAGTATTAGGC
	NP 1.25-	ACATTTTCACTTGTCCCGAATTTTTAATTACTC
P	P Start + B	GTGAAAATGTCTTTCCCCGAAGGCAAG
	M 2.12-	AGGACTCCATGTTTACTTGTCCC
M2	M2 Start + B	GACAAGTAAAGATGTCCAGAAGGAATCCCTG
	M2-1 end B-	TTGCACCTAATTACTGCTGTCACCC
L	L Start + B	GACCAATATGGACCCATCCAATGAG
	L end B-	CTTTATGGTCTATTTTGTGCTCAGTATGTACC

Table 4.3 Primers used to generate the support genes plasmids

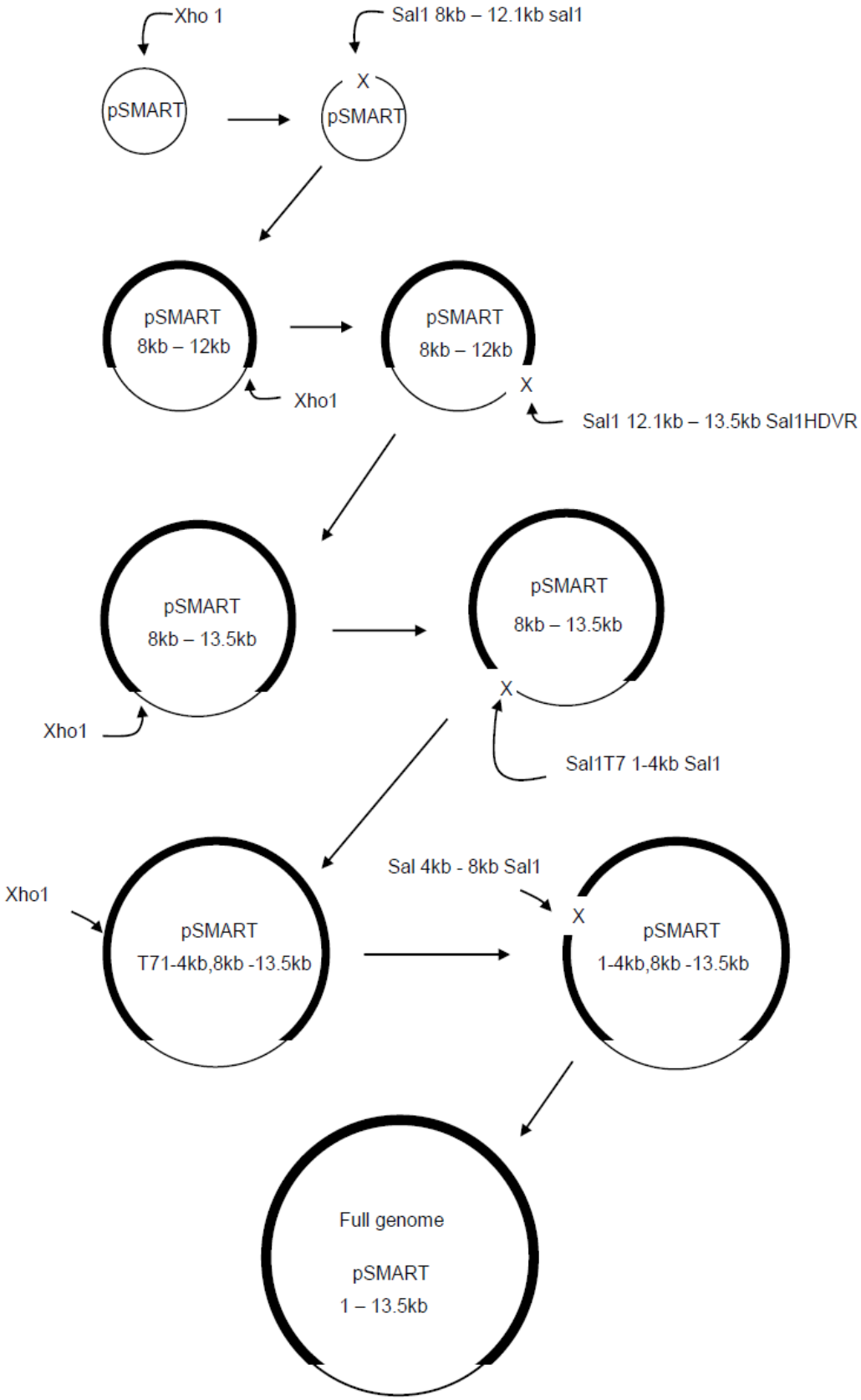


Figure 4.1 Schematic representation of the strategy used to construct a clone DNA copy of subtype B virus.

4.2.6 Recovery of viruses

Vero cells infected with a fowlpox recombinant virus expressing T7 polymerase were transfected initially with a cloned subtype A genome, together with subtype A support protein genes, and cloned subtype B support protein genes as they became available, using Lipofectamine 2000, under the same conditions and concentrations previously used for subtype A rescue (Naylor et al., 2004). Subsequently the cloned subtype B genome replaced the subtype A genome. Eventually subtype B components entirely replaced those from subtype A. In addition a subtype B genome copy was used with only subtype A components. Details are given in Table 4.8.

4.3 RESULTS

4.3.1 Determination and comparison of leaders and trailers sequences

Determined leader and trailer sequences are given in Table 4.4 and sequence chromatograms in Figure 4.2. For reference, leader and trailer sequences from a previously published subtype C virus are included in Table 4.4. For subtypes A and B, leaders or trailers sequences were always found to be in agreement for viruses within the same subtype.

The leader sequences of subtype A and B viruses were identical for the first 12 nucleotides and when compared to antigenomic trailer sequences, for subtype A they were identical for those first 12 nucleotides, whereas differences were found for subtype B. After position 12 similarities became minimal.

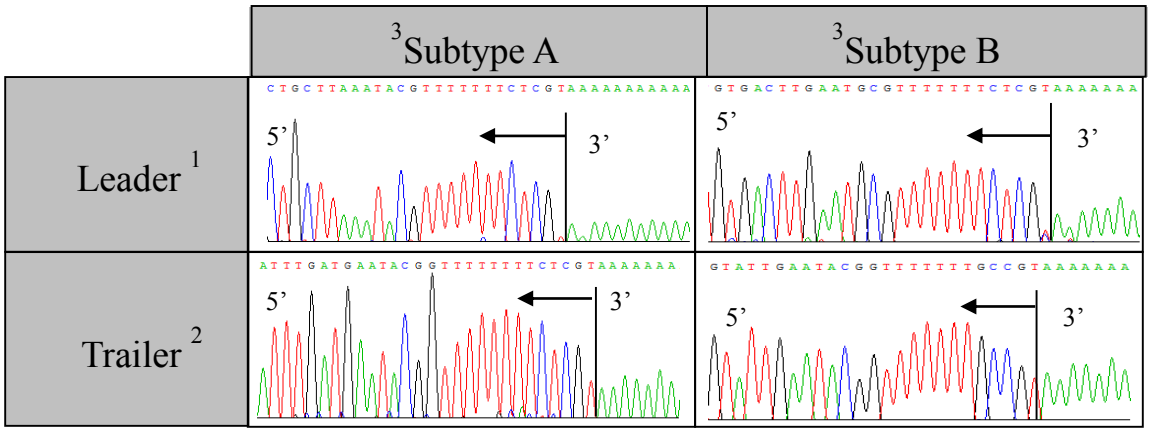
For the trailer, an antigenomic sequence from nucleotides 13-21 GGCAUAAGU was detected in all 3 subtypes. For all 3 subtypes the remaining 18-24 nucleotides

of the leader/trailer sequences up to the N start/L end were mainly comprised of apparently random Us and As and there was no obvious common sequence motif between the subtypes.

The 2 GGs normally assumed to be added to the virus leader due to use of a T7 promoter in RG derived viruses were never detected.

4.3.2 Determination and comparison of gene start and stop sequences

Determined mRNA sequence chromatograms for each gene are shown in Figure 4.3. Gene start and stop sequences for subtype A and B viruses are compared in Table 4.5 in genome sense (3'to 5') and include sequences predicted from a previously determined published subtype C virus full genome (accession number AY579780). All genes started with the sequence 3'CCCUGUUCA5' with the exception of F and SH genes of subtype B which started with 3'CCCCGUUCA5'. All gene stop signals started with UCA then had a variable sequence of generally 3 to 5 nucleotides after which followed between four and seven Us (which became the polyA tail), with the exception of the subtype A SH gene which had an 11 nucleotide separation but which still efficiently stopped transcription and led to polyadenylation. In the case of Germany A virus, sequence changes within this 11 nucleotide region led to absence of detectable monocistronic SH mRNA. This absence of detectable SH gene transcription termination would be assumed to prevent downstream G expression (Naylor et al., 2007; Whelan et al., 2004). Otherwise the subtype A and B transcription stop sequences were very similar as shown in Table 4.5 with a consensus for subtype A of UCAAU(A/U)A(A/U)UUUU and subtype B of UCAAUAU(A/U)UUUU.



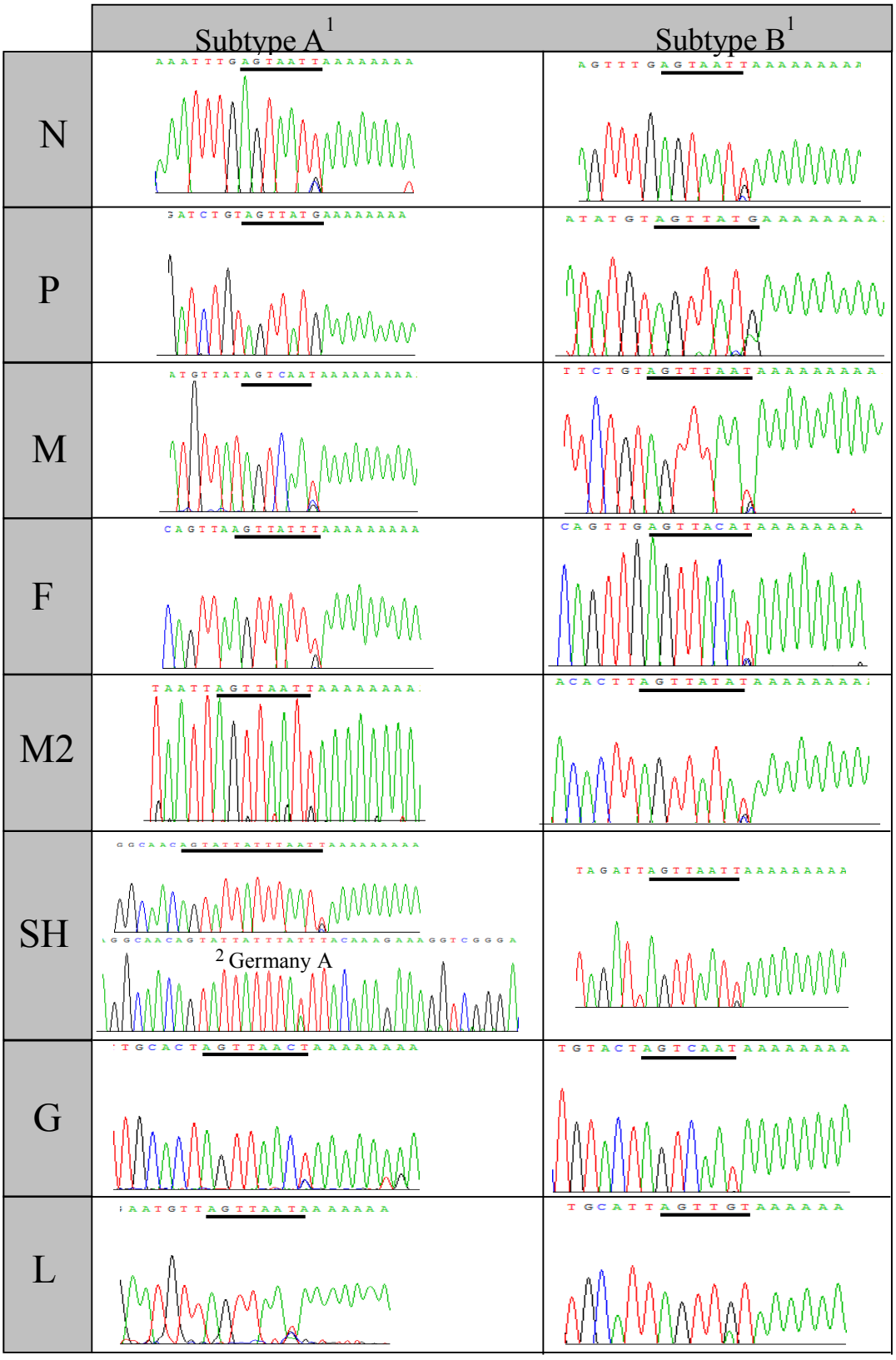
[1] [2] polyadenylated DNA copies of genomic sense leader
[3] polyadenylated DNA copies of antigenome sense trailer
Sequence common for all viruses of this subtype

Figure 4.2 Chromatograms of DNA copies of subtype A and B AMPV leader and trailer sequences

AMPV subtype	Leader and complimented trailer sequences for subtype A, B and C viruses
A leader	3′**UGCUCUUUUUUUGCAUAAAUUCGUC**.....N start 5′
A trailer[1]	3′**UGCUCUUUUUUUGGCAUAAGUAGU**.....L stop 5′
B Lead	3′**UGCUCUUUUUUUGCGUAAGUUCAG**.....N start 5′
B trailer[1]	3′**UGCCGUUUUUUUGGCAUAAGUUAU**.....L stop 5′
[2]C leader	3′**UGCUCUUUUUUUGCGUAUAUUCUG**.....N start 5′
[2]C trailer[1]	3′**UGCCGUUUUUUUGGCAUAAGUAGG**.....L stop 5′

[1] Antigenome sequence
[2] Not determined by the authors and based on accession AY579780

Table 4.4 Determined leader and trailer sequences for subtype A and B viruses, with published subtype C for reference



[1] Common terminal sequence found in all viruses sequenced except for subtype A, SH gene

[2] No monocistronic SH mRNA. Sequence displayed shows the region of discistronic SH-G mRNA

Figure 4.3 Chromatograms of DNA copies of subtype A and B AMPV leader and trailer sequences

Gene	AMPV subtype	Sequence from transcription start to subsequent transcription start
N	**A**	**CCCUGUUCAGUUUU -ORF+NCGE- UCA UUAA[2]UUUUUUUAUA**
	B	**CCCUGUUCAUUUU -ORF+NCGE- UCA UUAA[2]UUUUUAAG**
	C[1]	**CCCUGUUCA**CUUU -ORF+NCGE- UCA UUAAUUUUUUUAUA
P	**A**	**CCCUGUUCAUUGU -ORF+NCGE- UCA AUAC[2]UUUUUUA**
	B	**CCCUGUUCACUUU -ORF+NCGE- UCA AUAC[2]UUUUUUA**
	C[1]	**CCCUGUUCA**GUUU -ORF+NCGE- UCA AUUAUUUUUUG
M	**A**	**CCCUGUUCAGUUU -ORF+NCGE- UCA GUUA[2]UUUUUUAA**
	B	**CCCUGUUCAUUUG -ORF+NCGE- UCA AAUUA[2]UUUUUUAUA**
	C[1]	**CCCUGUUCA**CCUU -ORF+NCGE- UCA GUUCUAUUUGUGUCUCUCAUGUGAAUGGUUUAGUGUCAUU GUUAAAGCAAAAAUUGGGAGAGUAUCAAUAAUGGAUCGAACUAUAAUAAAUCUUUU UUAA
F	**A**	**CCCUGUUCAUCC -ORF+NCGE- UCA AUAAA[2]UUUUAA**
	B	**CCCCGUUCAUUU -ORF+NCGE- UCA AUGUA[2]UUUUUUCA**
	C	**CCCUGUUCA**CUUU -ORF+NCGE- UCA AUGAUUUUUUAA
M2	**A**	**CCCUGUUCACUUC -ORF+NCGE- UCA AUUAA [2]UUUUGGUUAAUUCGAUAUUCAGGUUUUUUCCCA**
	B	**CCCUGUUCAUUUC -ORF+NCGE- UCA AUAUA[2]UUUUUGUUAACUCGUGGGGGGGGCUUUUUUCUA**
	C[1]	**CCCUGUUCA**CUUC -ORF+NCGE- UCA AUUAUUUUUUAA
SH	**A**	**CCCUGUUCAGUAU -ORF+NCGE- UCA UAAUAAAUUAA[2]UUUUUCUUUCCAG**
	Germany A	**CCCUGUUCAGUAU -ORF+NCGE- UCA UAAUAAAUAAAUGUUUCUUUCCAG did not stop**
	B	**CCCCGUUCAGUUC -ORF+NCGE- UCA AUUAA[2]UUUUAGUCUUCUG**
	C[1]	**CCCUGUUCA**GUUG -ORF+NCGE- UCA AUAAAUUUUUAGUACUUAUACAGACCUGUCACGGUUCCGGUUC UUUUUGGUUGUGCUCUUGUCCACUAGGUUACUAAUUUUUGCUAGUCUCUUCCUUUU UG
G	**A**	**CCCUGUUCAUAGAGU-ORF+NCGE- UCA AUUGA[2]UUUUUACUUGUGUAUAUAUAGACUAUUAUUUUU UUGUGUAGUCUAUCAGAUUUUGUUAAUUUUCUUACUUUUGU**
	B	**CCCUGUUCAUAGGUC-ORF+NCGE- UCA GUUA[2]UUUUUCAUUGGAAAGUGUAGAUUUUAUUUCGUUUU UCUUCUUUUUUCUUCUUUCUUCCUUUCUUUCUUCUUCUUAUCGUGUGUUGUCUUUC CU**
	C[1]	**CCCUGUUCA**GUUG -ORF+NCGE- UCA AUUAAUUUUUCUU
L	**A**	**UCCUGGUUA -ORF+NCGE- UCA AUUA[2]UUUUU to Trailer**
	B	**CCCUGGUUA -ORF+NCGE- UCA AUA[2]UUUUUU to Trailer**
	C[1]	CCUGGUUCA -ORF+NCGE- UCA AUAAAUUUUU to Trailer

NCGE – non coding gene end
[1] Not determined by the authors and based on accession AY579780
[2] demonstrated start of polyadenylation in resulting mRNA

Table 4.5 Determined consensus gene stop signals for subtype A and B viruses, with predicted subtype C sequences based on database reference

	Subtype A					Subtype B					Subtype C[3]				
Nucleotide position	A	C	G	U		A	C	G	U		A	C	G	U	
1st	0	0	0	8[1]	U[2]	0	0	0	8	U	0	0	0	8	U
2nd	0	8	0	0	C	0	8	0	0	C	0	8	0	0	C
3rd	8	0	0	0	A	8	0	0	0	A	8	0	0	0	A
4th	5	1	0	2	A	6	0	1	1	A	6	0	1	1	A
5th	1	0	0	7	U	1	0	0	7	U	0	0	0	8	U
6th	4	0	0	4	A/U	4	0	1	3	A	3	0	1	4	U
7th	5	1	1	1	A	3	1	0	4	U	7	1	0	0	A
8th	4	0	0	4	A/U	4	0	0	4	A/U	3	0	0	5	U
9th	1	0	0	7	U	0	0	0	8	U	1	0	0	7	U
10th	1	0	0	7	U	0	0	0	8	U	0	0	0	8	U
11th	0	0	0	8	U	0	0	0	8	U	0	0	0	8	U
12th	1	0	0	7	U	0	0	0	8	U	0	0	0	8	U

[1] Black shading identifies the majority nucleotide at the given position within the eight gene stop signals
[2] Grey shading denotes the consensus stop signal for the given subtype
[3] Not determined by the authors and based on accession AY579780

Table 4.6 Determined consensus gene stop signals for subtype A and B viruses, with predicted subtype C sequences based on database reference

4.3.3 Comparison of viral protein sequences

Details of nucleotide identities, together with amino acid sequence identities and similarities for subtypes A, B and C are given for each gene in Table 4.7. Comparison of A, B and C sequences confirmed that subtype A and B proteins were more closely related to each other than they were to subtype C. Between subtypes A and B, those proteins expressed from transfected cloned DNA in the reverse genetics system, N, P, M2 and L, had amino acid similarities of over 80%, and this was also the case for M and F. In contrast when comparing either subtypes A or B to subtype C, the similarity fell to approximately 79% in the case of the L gene. For the nonessential genes SH and G (Naylor et al., 2004), amino acid similarities between subtypes A and B were much lower at 60% and 46% respectively and fell to approximately half those values when SH and G of either subtype was compared to subtype C.

Gene	Subtype A vs B			Subtype A vs C			Subtype B vs C		
	Nuc	aa		nuc	aa		nuc	aa	
N	76[1]	91[2]	97[3]	66	70	87	68	71	87
P	70	72	88	58	53	69	59	53	69
M	75	90	98	70	78	91	72	78	91
F	74	83	91	69	72	85	67	72	86
M2	78	89	96	64	71	88	65	73	86
SH	60	50	60	40	20	31	43	19	34
G	53	36	46	28	10	17	29	12	20
L	74	86	94	46	64	79	46	64	79

Shading denotes greater than 80% identity/similarity
[1] nucleotide identity
[2] amino acid identity
[3] amino acid similarity

Table 4.7 Nucleotide identities and predicted amino acid identities and similarities, comparing AMPV subtype A, B and C

4.3.4 Construction of subtype B reverse genetics system

4.3.4.1 pSMART plasmid vector preparation

pSMART plasmid vector was successfully circularized, transformed and isolated, after being liquid cultured. PCR and restriction enzyme analysis confirmed that the XhoI site was added in the cloning region.

4.3.4.2 AMPV-B full genome copy plasmids

Three amplicons covering the first 12kb of the AMPV subtype B vaccine genome were successfully generated by RT-PCR. The amplicon covering the last 1.5kb of the genome was obtained by PCR from a plasmid containing also the Hepatitis Delta Virus Ribozyme (HDVR) sequence. Each amplicon generated was flanked by SalI site.

After a series of ligation steps several FL cDNA plasmids was generated, but only one of them showed the correct restriction endonuclease (RE) pattern. The mapping PCRs on the positive plasmid generated 11 amplicons of the expected size. The full genome sequence obtained was aligned against RhinoCV sequence and the analysis didn't show any mutations. The plasmid was identified as vB.

4.3.4.3 Support genes plasmids

The amplicons of genes N, P, M2 and L were successfully obtained by RT-PCR or PCR and cloned into the same plasmids used for subtype A. Sequence analysis of the positive colonies showed that at least 1 plasmid per gene had the correct nucleic acid sequence.

4.3.5 Recovery of virus from AMPV full length copies

Combinations of cloned genes and genomes from both A and B subtypes are given in table 4.8, which shows that all combinations of subtype A and B components led to virus rescue.

Rescue attempt	Subtype B components	Subtype A components	Outcome
1	N	P M2 L genome	Virus recovered
2	N P	M2 L genome	Virus recovered
	N P M2	L genome	Virus recovered
3	N P M2 genome	L	Virus recovered
4	N P M2 L genome		Virus recovered
5	N P M2 L	genome	Virus recovered
6	genome	N P M2 L	Virus recovered

Table 4.8 Combinations of RG components used in virus rescue attempts and sucesfully rescued

4.4 DISCUSSION

In order to overcome the issues previously faced in to establish a reverse genetics (RG) system for avian metapneumovirus (AMPV) subtype B, in the present study was initially performed a comparison of subtype A and B to assess whether subtype A components could be partially or fully substituted.

Comparison of subtype A and B amino acid sequences of those proteins required for the RG system, N, M2 and L, showed very high levels of amino acid identity and similarity while P had a lower identity yet maintained 88% similarity. The fusion and matrix proteins were also highly similar. While SH and G genes identities were much lower, these genes are not required for virus replication in cell culture (Naylor et al., 2004) or turkeys (Naylor et al., 2010) so those differences were not considered an impediment to virus rescue. The subtype C sequences were more different, having polymerase identities and similarities with subtype A and B viruses of 64% and 79% respectively. These data suggested that subtype A and B viruses might be recovered from subtype A or B full-length genome copies using either subtype A or B support proteins. It is not clear whether in spite of the greater differences found for the subtype C polymerase, subtype A and B reverse genetics components might still recover virus from subtype C full length copies.

For similar viruses, the viral polymerase is known to recognise sequences in the leader and trailer which play a role in transcription, replication and genome encapsidation. (Whelan et al., 2004). The leader sequences of AMPV subtypes A, B and C and antigenome trailer of subtype A were identical for the first 12 nucleotides, whereas subtype B and C trailers had a 2 nucleotide mismatch. Beyond nucleotide 12, virus leaders did not match their trailers and furthermore no common sequence motif was seen when comparing between subtypes. In contrast within the

antigenome trailers of all three subtypes between nucleotides 13-21, a sequence of 3'GGCAUAAGU 5' was found. When later the NCBI database was searched for all available equivalent sequences (accession numbers HG934338 (subtype C, host duck), FJ 977568 (subtype C, host turkey), AB548428 (subtype B, host chicken), AY 590688 (subtype C host turkey)) this same sequence was always detected. While this sequence might be coincidental, it might also have some regulation role, perhaps in the replication of the antigenome copy in subtype A, B and C viruses. However further RG based studies would be required to substantiate such a hypotheses. But whatever the specific role of the sequence, or the extreme 12 nucleotides of the leaders and trailer, the similarity across subtypes would appear compatible with the notion of a subtype independent RG system.

Interestingly, while the use of a T7 promoter in the RG system would be expected to add two GG residues to the start of the antigenome copy which would be expected to be incorporated into the genome, and have sometimes been suspected of causing phenotypic differences between recombinant and original virus from which the DNA copy has been prepared, these were never detected. We therefore conclude that these are edited out at an early stage of the RG rescue. This is a helpful practical observation because while the T7 promoter is very useful in RG systems, it is sometime avoided because of this perceived implicit sequence addition.

A previously comprehensive minigenome investigation of gene start signal efficiencies showed that the CCCUGUUCA was most efficient and that the variant sequence of CCCCGUUCA found on subtype B SH and G proteins would be expected to reduce transcription of those genes (Edworthy & Easton, 2005). The L gene transcription start sequences proved an exception and minigenome studies showed a reduced transcription efficiency (Edworthy & Easton, 2005), as might be expected for a gene coding a protein needed in smaller amounts. Surprisingly gene starts of the otherwise more distantly related subtype C viruses (Brown et al., 2014)

like the subtype A viruses all used CCCUGUUCA, but again with the exception of the L gene. Clearly lack of gene start differences would means that gene start differences would not preclude a subtype independent RG system for AMPV.

Transcription stop sequences had not been previously reported for most AMPV genes. In general the sequences found for AMPV subtype A and B were in agreement with those found previously for respiratory syncytial virus (Harmon et al., 2001). Nonetheless, a study of seven recombinant subtype A viruses, each containing a GFP reporter gene at different intergenic regions had shown that GFP expression did not follow the accepted model and suggested that inefficient genome stop sequences may have been playing a role (Falchieri, 2012), as had already been found to affect protection induced by candidate vaccines only differing in the their SH gene ends (Naylor et al., 2007). Similarly in the current study it proved impossible to detect monocistronic SH mRNA in a German field strain which implies that the downstream G gene would be unlikely to be expressed, and may well help explain why in a previous study, the deletion of this G gene from the same virus only marginally reduced its protective capacity (Naylor et al., 2010). Nonetheless stop sequence differences between subtypes were not generally greater than those within subtypes, hence supported the notion of a subtype independent RG system.

The above data taken as whole suggested that for an AMPV RG system subtype A and B components might be fully interchanged. This proved the case because when subtype B components became available they proved able to be substituted for subtype A components in the RG systems – and once a fully subtype B RG had been produced, both subtype A and B full length genome copies were shown to efficiently produce virus when using either subtype A or B support proteins. This indicates that the viral polymerase of either subtype is able to attach to the leader and trailer, to recognise gene start and stop sequences, and that the key viral protein genes shared

sufficient functional similarity to support rescue. It remains uncertain as to whether subtype A/B components might be able to recover a full length subtype C copy, though this could easily be tested through collaboration between groups in possession of the different RG systems.

As a more practical point, the cloning of genome copies in bacterial plasmids offer considerable flexibility when compared to alternatives more able to handle difficult sequences such as cloning into bacterial artificial chromosomes or other larger viruses such as fowlpox or vaccinia. In this study the previously recognised ability of pSMART to accept influenza virus genome segments has been extended to include the full genomes of an AMPV genome exhaustively proven very difficult to otherwise clone. It would interesting to know the limits of this approach and perhaps explore potential with larger viruses such as coronaviruses.

5. MAKING AND TESTING SUBTYPE B AVIAN METAPNEUMOVIRUS IBV RECOMBINANTS

5.1 INTRODUCTION

Infectious bronchitis virus (IBV) is a coronavirus, belonging to the family Coronaviridae, subfamily coronaviranae; it is a major pathogen of chickens and it is distributed worldwide. The primary tissue of replication of the virus is the respiratory tract, but it has shown the ability to infect also kidneys, intestine and reproductive system (Cook *et al.*, 2012; Dhinakar Raj and Jones, 1997; Jackwood e de Wit, 2013). IBV has a positive sense non-segmented RNA genome, which can undergo recombination, thus leading to the emergence of new variants. New variants can have major disease significance if they are able to avoid protection induced by prevailing vaccines. (Capua et al., 1999; Cook et al., 1996b). The development of IBV-AMPV recombinant vaccines might help overcome those problems, because AMPVs field recombinants have never been demonstrated. Recombinant vaccines could also avoid the interference observed during co-vaccination of one-day-old chickens with two or more live attenuated vaccines (Cook et al., 2001; Ganapathy et al., 2005; Ganapathy et al., 2006)

Reverse genetics systems for avian matepneumovirus (AMPV) subtypes A and C have been developed (Naylor et al., 2004; Govinfarajan et al., 2006), allowing the generation of viruses with gene deletions, mutations and reporter gene insertion (Brown et al., 2011; Govindarajan et al., 2006; Ling et al., 2008; Naylor et al., 2010). The similar tropism of this virus for the tissues of the respiratory tract, pointed to

AMPV as an ideal candidate vector for the expression of IBV immunogenic proteins. In 2013 the first AMPV-A/IBV recombinant viruses were generated (Falchieri et al., 2013). Birds vaccinated with the recombinant viruses were challenged with an homologous IBV strain and some protection was seen. The low level of protective immunity might be attributed to the poor replication of the recombinant viruses observed in the trachea of the vaccinated birds. In recent years subtype B has shown to be the most detected in chicken farms, and infection studies suggested that this subtype replicates better in chicken compare to the other subtypes (Aung et al., 2008). For this reason AMPV subtype B was supposed to have better potential to deliver foreign genes.

Nuceocapsid (N) and the distal half of spike (S1) are believed to be the major immunogenic proteins of IBV: the N protein is able to stimulate a cell mediated immune response (Seo et alk., 1997), while the S1 gene is more more likely to stimulate the antibody production (Cavanagh et al., 1986: Mockett et al., 1984) In the present study, (N) and (S1) protein genes of IBV Massachusetts (Mass) were cloned into a plasmid containing the full length (FL) DNA genome copy of a commercial AMPV subtype B vaccine (RhinoCV-Intervet). The plasmids were transfected into VERO cells and the recombinant viruses were rescued. The recombinants generated were inoculated into specific pathogens free (SPF) chickens to determine their ability to induce protective immunity against a virulent IBV Mass strain. The protection was assessed by observation of the recovery of the cilial motility in the trachea of the challenged birds, as specified by the European Pharmacopoeia. Beyond the protective immunity, the ability of the recombinants to transcribe the exogenous genes, to replicate *in vivo* and to induce an immune response were also evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Addition of the cloning cassette

An XhoI restriction endonuclease (RE) site was added by site directed mutagenesis (SDM) (see methods chapter (mc)3.7) into the G-L intergenic region of a plasmid containing the full length DNA copy of an AMPV subtype B based on RhinoCV (Intervet) sequence. The cloning cassette was made by annealing two complementary primers which include a transcriptional start (GGGACAAGT), a Sal I restriction endonuclease site (GTCGAC) and a transcriptional stop (AGTCAATAAAAAA). The cloning cassette was ligated into the XhoI site (mc 3.6). This was transformed (mc 3.8) and the colonies were first checked by PCR (3.3) using primers matching on either side of the cassette site and by RE analyses of PCR products (mc3.9) before being grown in liquid culture (mc 3.8). The plasmids were extracted using Qiamp miniprep mini kit (Qiagen) and checked for integrity with RE (mc 3.9). PCR was performed across the G-L junction on apparently correct plasmids and the amplicons were sequenced (mc 3.5) to assess the orientation and the integrity of the cassette.

5.2.2 IBV S1 and N genes amplification and insertion

Infectious bronchitis virus (IBV) RNA was extracted from a Massachusetts (Mass) strain, using Qiamp viral RNA minikit (Qiagen), following the manufacturer's recommendation (mc 3.1). S1 and N genes were reverse transcribed (mc 3.2) and the cDNAs amplified using primers introducing XhoI site at the extremities (mc 3.3). The amplicons were ligated (mc 3.6) into the SalI site of the cassette. The plasmids obtained were transformed in competent cell (mc 3.8) and the colonies

were screened by PCR to check the correct orientation of the exogenous genes (mc 3.3). The positive colonies were liquid cultured (mc 3.8), the plasmids extracted (mc 3.1) and checked for integrity with RE (mc 3.9). The inserts genes were both amplified by PCR (3.3) and the sequences analysed (mc 3.5).

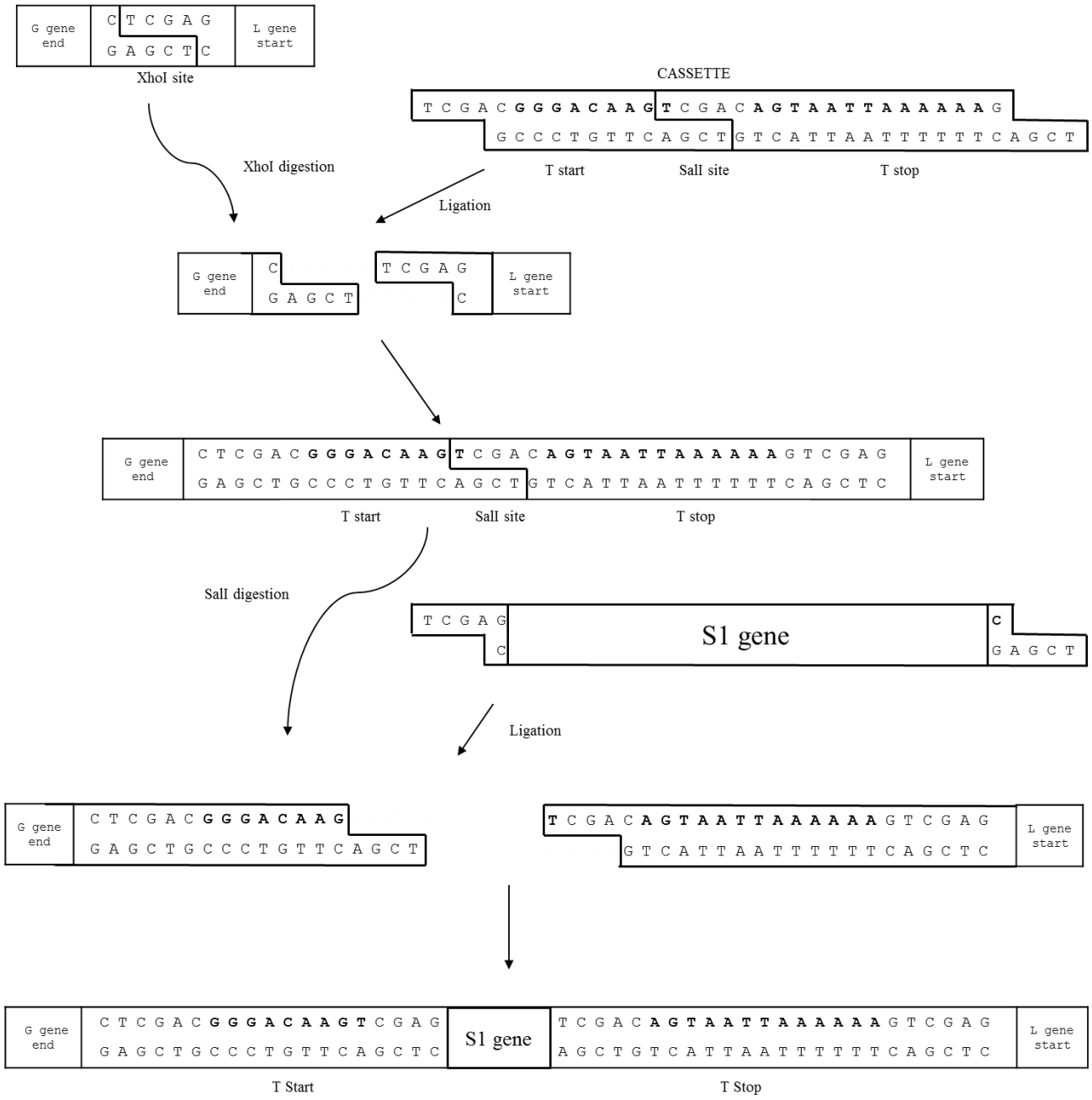


Figure 5.1 Schematic representation of the method used to insert the exogenous genes into subtype B DNA genome copy. The cloning casette was insert in the G-L intergenic region of the subtype B genome copy. The S1 and N genes were amplified by high fidelity RT-PCR, using RNA extracted from a Mass strain as template and the ligated into the casette.

Name	Sequence (5'…3')	Function
G-L XhoI +	CCTTTCACATCTAAAATAAAGCAAAAAGAACTCGAGAG AAGAAAGAAAGAAAGAAAGAAGAAGAACAGCACACAA C	XhoI site addition
G-L XhoI neg	GTTGTGTGCTGTTCTTCTTCTTTCTTTCTTTCTTTCTTCTC TCGAGTTCTTTTTGCTTTATTTTAGATGTGAAAGG	XhoI site addition
Cassette +	TCGACGGGACAAGTCGACAGTAATTAAAAAAG	Cloning cassette
Cassette neg	TCGACTTTTTTAATTACTGTCGACTTGTCCCG	Cloning cassette
N all neg	ACTAATGAGAATCACAATAATAAAAAGCACAG	N reverse transcription
N Xho start +	AAGGGACAACTCGAGCATGGCAAGCGGTAAGGC	N amplification XhoI site
N Xho end neg	CTTTTTTTCATAACTACTCGAGTCAAAGTTCATTCTCTCC	N amplification XhoI site
N end +	GATGATGAACCAAGACCAAAG	N Screening and mRNA
Dta-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTA	mRNA reverse transcription
Dtc-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTC	mRNA reverse transcription
Dtg-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTG	mRNA reverse transcription
Adaptneg	GCATCTCGAGGCTTGTGGCT	mRNA amplification
S1 end neg	CATCTTTAACGAACCATCTGG	S1 reverse transcription
S1 Xho Start +	GTGGTAAGTTACTGCTCGAGGATGTTGGTAACACCTCTT TTAC	S1 amplification XhoI site
S1 Xho end neg	AAGGGACAACTCGAGCATGGCAAGCGGTAAGGC	S1 amplification XhoI site
S1 end +	GCTGTTAGTTATAATTATCTAG	S1 Screening and mRNA
S1 675 +	GGATCACCTAGAGGCTTGTTAGC	S1 Sequencing
S1 765 neg	CACGATAGACAATAAACTTCTGCTTAAC	S1 Sequencing
B 7.46 neg	GGTATGGTCGTCCTATAATGCAAGATCC	N and S1 screening
GAB 4 +	GCTGATTGAGTGGTGCTGTACTAG	N, S1 and cassette sequencing
B 7.40 neg	GGAGTCAGGCAGATACACATTCACCG	N, S1 and cassette sequencing
G 7 +	GAAAAGACATTCAGTACATAC	mRNA amplification
SHf	TAGTTTTGATCTTCCTTGTTGC	*In vivo* replication assessment
SHr	GTAGTTGTGCTCAGCTCTGATA	*In vivo* replication assessment
MB-SH-r	FAM-CGCGATCATTGTGACAGCCAGCTTCACGATCGCG-Iowa Black FQ (Probe)	*In vivo* replication assessment

Table 5.1 List of the primers used in the study and their functions

5.2.3 Recovery of recombinant viruses

The plasmids containing the modified sequence were transfected on VERO cells following the methodology described in paragraph 4.6. In the presence of cytopathic effect (CPE), the virus rescue was further confirmed by RT-PCR of viral mRNA and the inserted gene was sequenced (mc 3.2; 3.3; 3.5). Rescued viruses were further passaged in Vero cells to produce a sufficient yield for protection studies. Viruses were titrated in 48 well plates containing Vero cell monolayers. CPE end points were observed and titres were calculated.

5.2.4 S1 and N genes transcription

In order to evaluate the transcription of the exogenous genes, the RNA was extracted from the VERO cells (mc 3.1) and amplified by RT-PCR following the protocol previously described in paragraph 4.2.3, except that primers within the genes were S1 end+ and N end+ for S1 and N genes respectively.

5.2.5 *In vivo* trial

Fifty Specific Pathogens Free (SPF) chickens were divided in 5 groups and each group was housed in a biological isolators. At 1 day-of-age chickens of groups 1-4 were vaccinated by eye drop, with 4 $\log_{10}$ TCID50 of $B_{N\ G\text{-}L}$, $B_{S1\ G\text{-}L}$, vB and a commercial available Mass vaccine respectively (Table 5.3). Birds of group 5 remained unvaccinated. All the vaccinated birds and half of the unvaccinated birds were challenged 21 days post vaccination (d.p.v.) with a dose of 4 $\log_{10}$ TCID50 of a virulent Mass type strain. Half the birds of each group were humanely killed 5 days post challenge (d.p.c.) and tracheas collected. At 6 d.p.c. the remaining birds were humanely killed and the tracheas were collected.

5.2.6 Serology

Chickens from all the groups were bled 18 days post vaccination (d.p.v.). The sera were tested by ELISA using a kit direct against AMPV subtype B antibodies (Biocheck).

5.2.7 Recombinants replication *in vivo*

To assess the replication *in vivo* of the AMPV-B recombinants, tracheal swabs were collected from the birds 5 d.p.v. The RNA was extracted from the swabs (mc 3.1) and amplified by real time RT-PCR following the protocol developed by Cecchinato et al. (2013). All reactions were carried out on LightCycler 480 (Roche Diagnostic) using a Superscript III Platinum One-Step Quantitative RT-PCR kit (Life Technologies). Primers and probes used were listed in table 5.1. A positive control with known titre was added at each reaction.

5.2.8 Determination of tracheal cilia activity

On days 5 and 6 post challenge (d.p.c.) the tracheas of 5 birds of each group were collected. The tracheas were cut in 1mm transverse section and for each trachea, 3 upper, 4 middle and 3 lower sections were observed using low power microscopy to determine activity of the cilia.

5.3 RESULTS

5.3.1 IBV recombinants AMPV-B construction

The N and the S1 amplicons generated by RT-PCR were ligated into the cassette in two separate reactions. The constructs containing the N gene were identified as $B_{N\ G\text{-}L}$, while the constructs containing the S1 gene were identified as $B_{S1\ G\text{-}L}$. Only one $B_{N\ G\text{-}L}$ construct had no mutations in the N gene sequence and showed the expected

restriction endonuclease (RE) profile. 5 correct plasmids containing the S1 gene were identified.

5.3.2 Recovery of the recombinant viruses

The recovery of both the viruses was confirmed by the presence of cytopathic effect (CPE) on the VERO cell monolayer at 5 day post infection (d.p.i.) on the second passage for both the recombinants. Only one of the $B_{S1\ G\text{-}L}$ constructs was recovered. As further confirmation of the virus recovery, amplicons of the expected size were obtained by RT-PCR targeting the AMPV viral mRNA. After 3 passages on VERO cell the recombinant virus's titres were sufficient for vaccination with 4 log10 dose per bird in 100ul eye drop (Table 5.2). The sequencing of the insert genes did not show any mutations. RT-PCR targeting the mRNA demonstrated that the recombinant viruses actively transcribed the exogenous genes.

Clone	**IBV Mass insert gene**	**Titre on VERO cells**
vB	None	5.5 $\log_{10}$/ml
B_N	N	5.3 $\log_{10}$/ml
B_{S1}	S1	5.3 $\log_{10}$/ml

Table 5.2 Summary of the viruses rescued and their titre in vitro

5.3.3 Serology

Antibodies against AMPV were generally not detected in the birds vaccinated with the recombinant vaccines, although one bird vaccinated with $B_{S1\ G\text{-}L}$ showed seroconversion. Poor antibody response was observed also in birds vaccinated only with vB: seroconversion was detected in only 3 birds (Table 5.3).

5.3.4 Recombinants replication *in vivo*

While AMPV qRT-PCR on trachea swabs showed replication of the recombinant in most birds, the replication was negligible. A similar replication was observed for vB (Table 5.3).

5.3.5 Tracheal motility following challenge

At 5 day post challenge (d.p.c.), none of the birds vaccinated with the recombinant viruses showed cilial motility. On 6 d.p.c. recovery was observed in a few sections of the trachea from birds vaccinated with $B_{N\ G.L}$ and $B_{S1\ G\text{-}L}$ (Table 5.3). Birds vaccinated with a commercially available Mass vaccine showed full protection. Unvaccinated/ challenged birds and birds vaccinated with the AMPV vector showed no cilial motility, while unvaccinated/unchallenged birds showed full motility (Table 5.3).

Groups	**Real-Time (5 d.p.v.)**		**Serology (18 d.p.v.)**	**% TOC beating**	
	Positve	I.D. mean		5 d.p.c.	6 d.p.c.
B_N	10/10	1	0/10	0	6
B_{S1}	8/10	10	1/10	0	8
vB	9/10	10	3/10	0	0
Mass	n.d.	n.d.	n.d.	100	100
Unvacc/challenged	0/5	0	0/5	0	0
Unvacc/unchallenged	0/5	0	0/5	100	100

Table 5.3 Effects of vaccination with three subtype B recombinats on virus replication, antibody response to AMPV and % TOC beating after challenge with IBV.

5.4 DISCUSSION

Recombinants of avian metapneumovirus (AMPV) subtype B were generated for the first time. The recombinants contained the N protein and the S1 protein of infectious bronchitis virus (IBV), which are believed to be the major immunogenic proteins of the virus. The recombinants showed to be stable after passage *in vitro* and to be able to transcribe the IBV exogenous genes. *In vitro* the recombinants reached titre comparable to those generally observed for VERO cells adapt AMPVs (Naylor and Jones, 1993). Virus titre is of great importance in vaccine developments because if sufficient titre per cell culture area cannot be achieved, the vaccine candidate will be rejected on technical cost and grounds.

When inoculated in one-day-old SPF chickens subtype recombinants conferred negligible protection at the challenge against an homologous IBV strain. The recombinants replicated poorly in the respiratory tract of the birds, the primary replication site of both AMPV and IBV. Poor replication was also observed for the vector without any exogenous genes, suggesting that the insertion of foreign genes did not affect the virus viability. The poor replication in the upper respiratory tract is likely to have prevented the induction of protection against IBV. Using the reverse genetics system it would be possible to verify this hypothesis modifying the sequence of the vector in order to increase the replication *in vivo*.

No significant differences in term of protection, replication and antibody response were observed between the two recombinants. In a previous study the subtype A recombinant expressing the QX N protein protected better than that expressing QX S1 (Falchieri et al., 2013). This difference was not seen using the current Mass recombinants. This might be due to the differences between QX and Mass proteins or differences between the challenge models. Alternatively the low level of

replication of the viruses may give insufficient inserted gene expression to provide stimulation of the immune response. Again, this could be tested by modifying the viruses by reverse genetics to increase their replication *in vivo*.

Despite the low replication and the poor protection, AMPV-B remained a potentially promising vector. The subtype B virus was shown to be able to accept almost 2000 extra nucleotides without affecting virus viability and the upper limits have not been yet determined. Furthermore, AMPV recombinants was shown to be stable after *in vitro* passage. This contrasts with single strand positive sense viruses such as IBV where just few passages results in sequence mutations and recombination events (Cavanagh, 2007).

To conclude, AMPV subtype B was shown to be a suitable vector for the expression of IBV immunogenic proteins, but, as the recombinants tested were able to induce negligible protection, further studies need to be done in order to generate efficient vaccine.

6. IMPROVING THE REPLICATION *IN VIVO* OF SUBTYPE B AVIAN METAPNEUMOVIRUS IBV RECOMBINANTS

6.1 INTRODUCTION

Previously reverse genetics (RG) systems for the subtype A and C (Govindarajan et al., 2006; Naylor et a., 2004) were made and more recently a similar system for subtype B has been developed (chapter 4).

Using subtype A RG system, the ability of AMPV to accept and express exogenous genes has been demonstrated, thus identifying this virus as an ideal candidate vector for the expression of immunogenic proteins of other poultry respiratory viruses (Falchieri et al., 2013). Subtype A and B AMPV/IBV stable recombinants has been generated, but they all replicated poorly *in vivo* and conferred negligible protection at the challenge (Falchieri et al., 2013: chapter 5). A correlation between the poor replication and the negligible protection was suggested and it was hypothesised that increasing the recombinant's replication in the trachea might improve the ability to induce protective immunity.

The recombinant viruses developed in the previous chapter were based on the sequence of a commercial available vaccine (RhinoCV-Intervet). Another commercial available AMPV subtype B vaccine, Nemovac (Merial), was shown to replicate better in chicken (private communication). In the present study the full genome sequences of Nemovac and RhinoCV were compared and all the coding changes were identified. Most of the coding changes were corrected and a plasmid containing a hybrid AMPV subtype B FL genome sequence was generated. The

sequences of the S1 and N genes of a Massachusetts (Mass) IBV strain were cloned into the G-L intergenic region of the chimera FL cDNA.

To further increase protection, another approach has also been followed. Several studies indicate that the co-expression of Interleukins can enhance the efficacy of live vaccine (Göbel et al., 2003; Winfried et al., 2004). Interleukin-18 (IL-18) plays an important role in the inflammatory reaction in chickens, stimulating the release of interferon ɣ (IFN-ɣ) (Schneider et al., 2000). Chicken's IL-18 gene was amplified by PCR and cloned in a second cloning cassette added downstream the S1 or the N gene of the chimera FL cDNA.

Plasmids generated were transfected into Vero cells. The rescued viruses were tested for the ability to induce protective immunity against challenge with a homologous IBV virulent strain. The ability of the recombinants to replicate *in vivo* and to transcribe and express the exogenous genes was also evaluated.

6.2 MATERIALS AND METHODS

6.2.1 Subtype B vaccines comparison

RhinoCV and Nemovac RNA was extracted using Qiamp Viral RNA minikit (Qiagen) following the manufacturer's recommendation (see methods chapter (mc) 3.1). The RNA was reverse transcribed (mc 3.2) and amplified by PCR (mc 3.3). All the amplicons were purified and sequenced by Source Bioscience Sequencing (Cambridge UK) and the full genome sequences generated were analysed using Bioedit Sequence Alignment Editor. The primers used are listed in table 6.1. The full genome sequences of the virus were translated and analysed using Generunner.

6.2.2 Chimera AMPV-B construcion

Nemovac RNA was extracted (mc 3.1), reverse transcribed (mc 3.2) and the cDNA was amplified (mc 3.3) to cover the region from the beginning of the F gene, to the end of the G gene. The amplicons were used as megaprimers in site directed mutagenesis (SDM) to convert the RhinoCV FL cDNA sequence (mc 3.7). Failures in both RT-PCR and SDM forced the adoption of a new approach. The sequence to be modified was further analysed and a region reach in G-C content was identified at the end of the G gene, suggesting a possible role in the poor amplification and genome modification performances. Thus the cDNA was amplified in two separated PCRs (mc 3.3): the first covered the F, M2, SH genes and the beginning of the G gene, the second one covered the rest of G gene, including the G-C reach region. An SDM was performed on the plasmid containing the RhinoCV sequence, using the first amplicon as megaprimer (m 3.7). The SDM products were transformed on STB12 cells, grown on liquid culture (mc 3.8) and plasmids were purified and checked for integrity by restriction endonuclease (RE) analysis (mc 3.9). The modified region was amplified and sequenced (mc 3.5). On plasmids showing the expected sequence a second SDM was performed, using the second PCR amplicons as megaprimer (mc 3.7). Firstly the modified region and then the full plasmids were sequenced and analysed (mc 3.5).

6.2.3 IBV S1 and N genes amplification and insertion

The N and the S1 genes were amplified and cloned into the chimera plasmid following the same protocol described in paragraph 5.2.2. Briefly, an XhoI site was introduced in the G-L intergenic region by SDM (mc 3.7) into which the cloning cassette was ligated (mc 3.6). The S1 or the N gene were then inserted into the

cloning cassette (mc 3.6). After each ligation step, the cloning site and the insert genes were sequenced (mc 3.5).

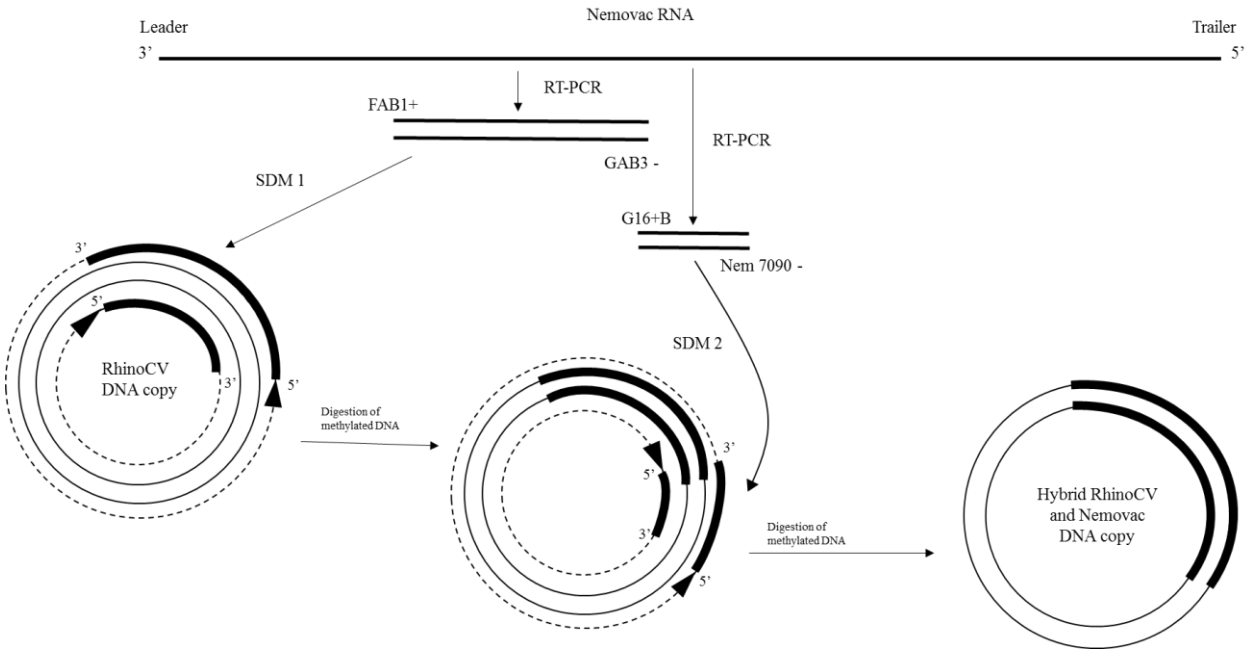


Figure 6.1 Schematic representation of the method used to convert a RhinoCV DNA full genome copy. RhinoCV plasmid was modified by site directed mutagenesis in two steps by two high fidelity RT-PCR amplicons, generated using RNA extracted from Nemovac as template.

6.2.4 Interleukin-18 (IL-18) gene amplification and insertion

The Interleukin 18 (IL-18) was provided by Ceva Japan K.K. and it was amplified by PCR (mc 3.3) using primers introducing XhoI site at the extremities of the amplicons. SDM (mc 3.7), using primers containing a SalI site flanked by a transcriptional start and a transcriptional stop, was performed on the chimera plasmid containing the S1 gene sequence. The amplicons of IL-18 were ligated into the SalI site of the cloning cassette (mc 3.6). Plasmids containing the two exogenous genes were transformed on STB12 cells, liquid cultured (mc 3.8), purified and cut with RE (mc 3.9). Both the insert genes were amplified and sequenced (mc 3.5).

A different approach was used to generate the N+IL-18 chimera plasmid, as described in figure 6.2. Primers were designed to remove by PCR (mc 3.3) the S1 gene from the plasmid containing the S1 and IL-18 genes. The linearized plasmid was ligated (mc 3.6) with the N gene amplicons and transformed on STB12 cell (mc 3.8). Purified plasmids were screened by PCR (mc 3.3) and checked for integrity with RE (mc 3.9). The N and the IL-18 genes were amplified and sequenced (mc 3.5).

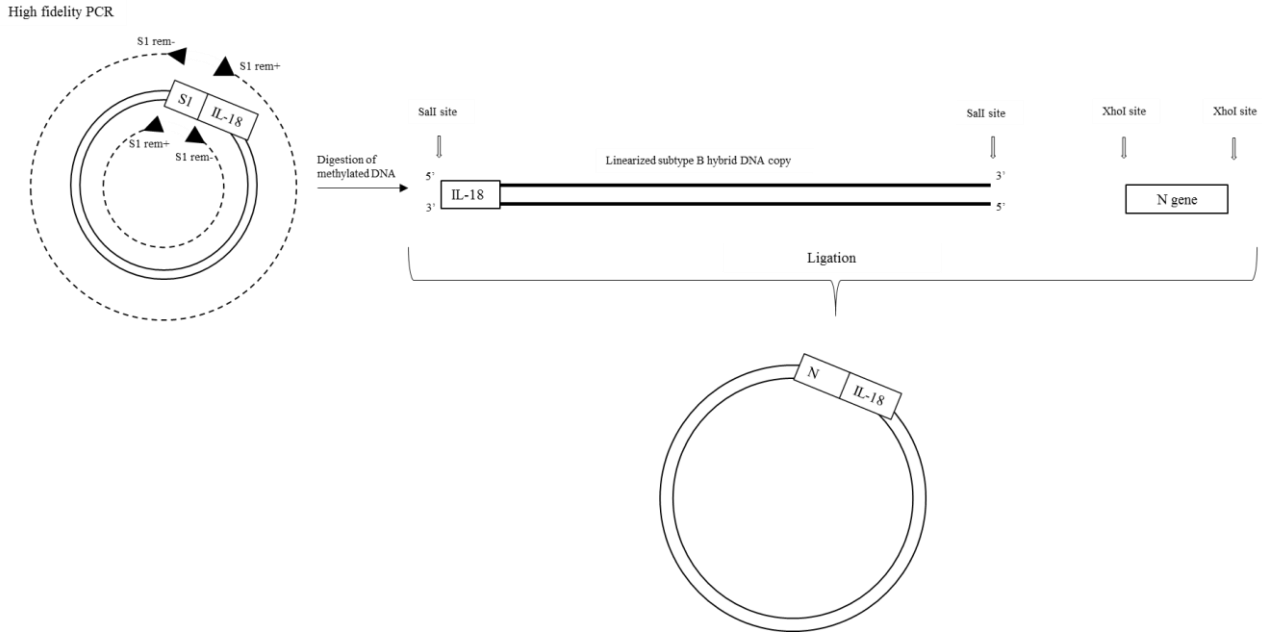


Figure 6.2 Schematic representation of the method used to insert the N gene in the construct containing the S1 and the IL-18 genes. The S1 gene was removed by high fidelity PCR and the linearized PCR product was ligate with the N gene, previosly amplify by high fidelity RT-PCR, using RNA extracted from an IBV Mass strain.

6.2.5 Recovery of recombinant viruses

The plasmids generated (Table 6.4) were transfected into VERO cells following the procedure described in paragraph 4.6. The observation of cytopathic effect (CPE) was used as confirmation of the presence of the virus. As further confirmation, the

viral RNA was extracted (mc 3.1) from the cell sheets showing CPE and a RT-PCR (mc 3.2; 3.3) directed against AMPV viral mRNA was performed. The insert genes were sequenced in order to exclude any corruptions or mutations (mc 3.5). Rescued viruses were further passaged and titrated on VERO cells ready for protection studies.

RT-Primers	
Name	**Sequence**
Ac le A tr 15	ACGAGAAAAAAACGC
M2 Start +	GATGTCTAGGCGAAATCCC
L 2 +	GAAAGGGAACTAAGTGTAGG
L end B neg	CTTTATGGTCTATTTTGTGCTCAGTATGTACC
PCR primers[1]	
Name	**Sequence**
B.003 +	ACAAGTCACAATAGAAAAGAGA
NP 1.25 neg	GACATTTTCACTTGTCCCGAATTTTTAATTACTC
NAB 2 +	CTAGATCCCTCAAAGAGAGCAACAAG
B 2680 neg	CTAGATCCCTCAAAGAGAGCAACAAG
MAB 1 +	GGACAACAACCCTGCAAAACTGAC
FAB 4 neg	CTCAACTGATGTAGCCCATGTTGC
FAB 3 +	CTAATGACTTACTGGACATAGAGGTTAAGAG
G 3 NEG	ACTAGTACAGCACCACTC
G 15 + B	GCAAGACGACCGACCAGAGAC
B 7840 neg	CATCTCTGCAGCATTGGACATATCG
LAB 1 +	CTGGAAGTGTCACAGACCAGTGC
LAB 4 neg	CCCCACACTTAATTCCCTTTCTTTTCC
LAB 3 +	CGTGTACTAGAGTTTTACTTGAAGGATGC
LAB 9 neg	CAAGTTAATGTCCTCATTTCCAAATCTCTCAC
LAB 8 +	GTAGACCGATGGAGTTTCCTTCATCAG
L end B neg	CTTTATGGTCTATTTTGTGCTCAGTATGTACC
Sequencing primers	
Name	**Sequence**
MAB 3 +	GAGAGCTTAGGGAAAATATGCAAAACATGG
FAB 1 +	GCTAAAACAATAAGATTAGAAGGGGAGGTG
FAB 2 +	ATGACTATGTGTTCTGTGATACTGCAGC
M2 AB 1 +	GAATCCAGCAAATCTCATAAACAGTCTCAAG
GAB 3 neg	GTATCTCCCTGACAAATTGGTCCTG
GAB 1 +	GGCTTGACGCTCACTAGCACTATTG
GAB 4 +	GCTGATTGAGTGGTGCTGTACTAG
B 7.40 neg	GGAGTCAGGCAGATACACATTCACCG
B 7.90 neg	ATTCCAACAGCTTTTACGGAGG
LAB 2 +	GATATGTCCAATGCTGCAGAGATG
LAB 6 +	GGAGACCCTGTTGTTGTGTATAGGAG
LAB 7 +	CATTGATAGAGCAGTTCATATGATGTTGCTC
LAB 10 +	CATTGATAGAGCAGTTCATATGATGTTGCTC

[1] All primers used to generate the PCR products, were also used for the full genome sequencing

Tabella 6.1 List of the primers used to sequence the full AMPV subtype B genome

Name	Sequence (5'…3')	Function
FAB 1 +	GCTAAAACAATAAGATTAGAAGGGGAGGTG	Nemovac amplification
GAB 3 neg	GTATCTCCCTGACAAATTGGTCCTG	Nemovac amplification
G 16 + B	CCTTACATCGAGGACAGTCAAC	Nemovac amplification
Nem 7090 neg	GACTAGGATTGTAAGTTCCTACCTGG	Nemovac amplification
G-L XhoI +	CCTTTCACATCTAAAATAAAGCAAAAAGAACTCGAGAG AAGAAAGAAAGAAAGAAAGAAGAAGAACAGCACACAA C	XhoI site addition
G-L XhoI neg	GTTGTGTGCTGTTCTTCTTCTTTCTTTCTTTCTTTCTTCTC TCGAGTTCTTTTTGCTTTATTTTAGATGTGAAAGG	XhoI site addition
Cassette +	TCGACGGGACAAGTCGACAGTAATTAAAAAAG	S1 and N cloning cassette
Cassette neg	TCGACTTTTTTAATTACTGTCGACTTGTCCCG	S1 and N cloning cassette
N all neg	ACTAATGAGAATCACAATAATAAAAAGCACAG	N reverse transcription
N Xho start +	AAGGGACAACTCGAGCATGGCAAGCGGTAAGGC	N amplification XhoI site
N Xho end neg	CTTTTTTTCATAACTACTCGAGTCAAAGTTCATTCTCTCC	N amplification XhoI site
N end +	GATGATGAACCAAGACCAAAG	N Screening and mRNA
S1 end neg	CATCTTTAACGAACCATCTGG	S1 reverse transcription
S1 Xho Start +	GTGGTAAGTTACTGCTCGAGGATGTTGGTAACACCTCTT TTAC	S1 amplification XhoI site
S1 Xho end neg	AAGGGACAACTCGAGCATGGCAAGCGGTAAGGC	S1 amplification XhoI site
S1 end +	GCTGTTAGTTATAATTATCTAG	S1 Screening and mRNA
S1 675 +	GGATCACCTAGAGGCTTGTTAGC	S1 Sequencing
S1 765 neg	CACGATAGACAATAAACTTCTGCTTAAC	S1 Sequencing
S1 ins +	CACTAATGGAACATAGTTATTAAAACGTTAACGGGACA AGTCGAC	IL-18 cloning cassette
S1 ins -	CGGATATTTCCATACTTGTCCCTGTTTTTCTCGACTTTTT GTCGA	IL-18 cloning cassette
IL-18 Xho +	CTTCCAGAGATTGGCTCGAGGATGAGCTGTG	IL-18 amplification XhoI site
IL-18 Xho neg	GTTCGAGGATTCTCGAGATATATCATAGGTTG	IL-18 amplification XhoI site
IL-18 285 +	GCCTGTTGCATTCAGCGTCC	IL-18 screening and mRNA
IL-18 345 neg	CGAACAACCATTTTCCCATGCTC	IL-18 sequencing
S1 rem Sal +	GTTTTACATTGTCGACACTAATGGAACATAGTTATTAAA CG	S1 removal
S1 rem Sal neg	GTTACCAACATCGTCGACTTCTTGTCCCTTTCTTCTTTTT GC	S1 removal
B 7.46 neg	GGTATGGTCGTCCTATAATGCAAGATCC	Insert genes screening
GAB 4 +	GCTGATTGAGTGGTGCTGTACTAG	Insert genes sequencing
B 7.40 neg	GGAGTCAGGCAGATACACATTCACCG	Insert genes sequencing
Dta-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTA	mRNA reverse transcription
Dtc-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTC	mRNA reverse transcription
Dtg-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTG	mRNA reverse transcription
Adaptneg	GCATCTCGAGGCTTGTGGCT	mRNA amplification
G 7 +	GAAAAGACATTCAGTACATAC	mRNA amplification
SHf	TAGTTTTGATCTTCCTTGTTGC	*In vivo* replication assessment
SHr	GTAGTTGTGCTCAGCTCTGATA	*In vivo* replication assessment
MB-SH-r	FAM-CGCGATCATTGTGACAGCCAGCTTCACGATCGCG-Iowa Black FQ (Probe)	*In vivo* replication assessment

Table 6.2 List of the other primers used in the study and their functions

6.2.6 Exogenous genes transcription

The ability of the rescued viruses to transcribe the exogenous genes was evaluated by RT-PCR (mc 3.2; 3.3). The RNA was extracted from the VERO cell (mc 3.1). The protocol described in paragraph 4.2.3 was adopted, except that primers within those genes were S1 end+, N end+ and IL-18 285+for S1, N and IL-18 genes respectively.

6.2.7 Exogenous proteins expression

S1 and N protein expression was assessed using immunofluorescence (IF) on AMPV recombinant infected VERO monolayers. A polyclonal chicken antiserum (GD) anti Mass was used to evaluate S1 expression and a monoclonal mouse antibody (Biozol) was used to evaluate the N expression. Suitable FITC conjugated anti chicken/mouse antibodies (Sigma) were used according to manufacturers' protocols to enable visualization of specific S1/N proteins.

6.2.8 Sequences comparison of two $cB_{N\ G\text{-}L}$ plasmids

Mapping PCR was performed on two plasmids containing the N gene ($cB_{N\ G\text{-}L}$). One, lab code 6_{29}, was successfully rescued, while the other, lab code 6_{27}, wasn't rescued. The amplicons generated were sequenced and aligned (mc 3.5). The full genome sequences obtained were compared and analysed (mc 3.5).

6.2.9 *In vivo* trial

Seventy Specific Pathogens Free (SPF) chickens were divided in 7 groups and each group was housed in biological isolators. At 1 day of age chickens of groups 1-5 were vaccinated by eye drop, with 4 $\log_{10}$ TCID50 of $cB_{N\ G\text{-}L}$, $cB_{S1\ G\text{-}L}$, $cB_{S1+IL\text{-}18\ G\text{-}L}$, cvB and a commercially available Mass type vaccine respectively (Table 6.5). Group 6 were vaccinated with a higher dose of $cB_{N\ G\text{-}L}$ (5 $\log_{10}$ TCID50). Birds of group 7 remained unvaccinated. All the vaccinated birds and half of the unvaccinated birds were challenged 21 days post vaccination (d.p.v.) with a dose of 4 $\log_{10}$ TCID50 of a virulent Mass type strain. Half the birds of each group were humanely killed 5 days post challenge (d.p.c.) and the tracheas were collected. On 6 d.p.c. the remaining birds were humanely killed and the tracheas were collected.

6.2.10 Recombinants replication *in vivo*

The replication *in vivo* of the AMPV-B chimeras were assessed by real time RT-PCR following the protocol developed by Cecchinato et al. (2013). Tracheal swabs were collected from the birds 5 d.p.v.. The RNA was extracted from the swabs using Qiamp viral RNA mini kit (Qiagen): all reactions were carried out on a LightCycler 480 (Roche Diagnostic) using a Superscript III Platinum One-Step Quantitative RT-PCR kit (Life Technologies). Primers and probes used were listed in table 6.2. A positive control with known titre was added at each reaction.

6.2.11 Determination of tracheal cilia activity

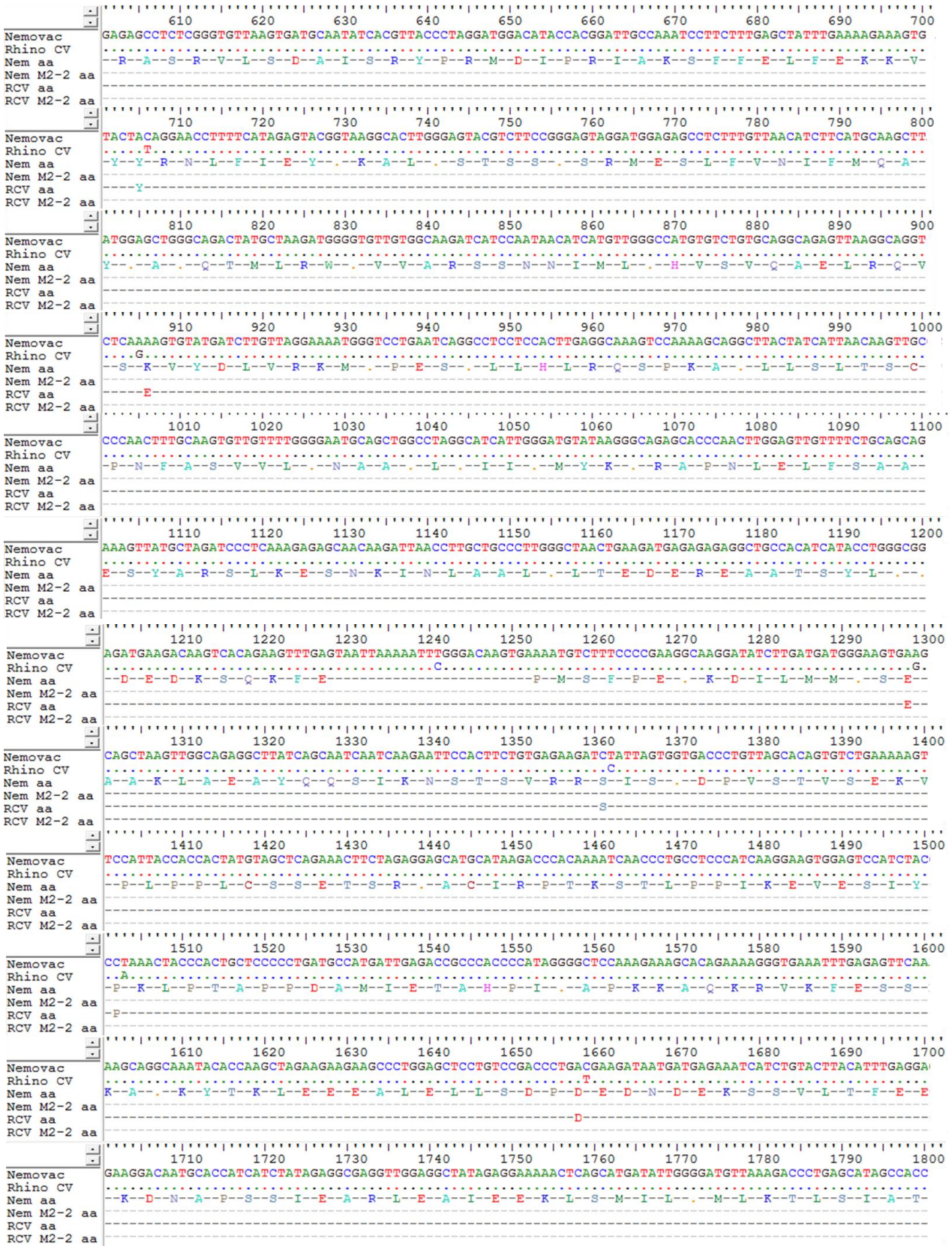
On days 5 and 6 post challenge (d.p.c.) the tracheas of 5 birds of each group were collected. The tracheas were cut in 1mm transverse section and for each trachea, 3

upper, 4 middle and 3 lower sections were viewed using low power microscopy to determine activity of the cilia.

6.3 RESULTS

6.3.1 Subtype B vaccines comparison

Eight amplicons of the expected size were generated by RT-PCR both for RhinoCV and Nemovac. The comparison of the sequences revealed the presence of 133 nucleotide changes between the two viruses, of which 41 were coding (Table 6.3). The majority of the coding changes (27) were observed in the F, SH, M2 and G genes, with G showing the lowest similarity 13 coding changes.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TACTACAGGAACCTTTTCATAGAGTACGGTAAGGCACTTGGGAGTACGTCTTCCGGGAGTAGGATGGAGAGCCTCTTTGTTAACATCTTCATGCAAGCTT
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1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
CCCAACTTTGCAAGTGTTGTTTTGGGGAATGCAGCTGGCCTAGGCATCATTGGGATGTATAAGGGCAGAGCACCCAACTTGGAGTTGTTTTCTGCAGCAG
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AAAGTTATGCTAGATCCCTCAAAGAGAGCAACAAGATTAACCTTGCTGCCCTTGGGCTAACTGAAGATGAGAGAGAGGCTGCCACATCATACCTGGGCGG
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CAGCTAAGTTGGCAGAGGCTTATCAGCAATCAATCAAGAATTCCACTTCTGTGAGAAGATCTATTAGTGGTGACCCTGTTAGCACAGTGTCTGAAAAAGT
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
TCCATTACCACCACTATGTAGCTCAGAAACTTCTAGAGGAGCATGCATAAGACCCACAAAATCAACCCTGCCTCCCATCAAGGAAGTGGAGTCCATCTAC
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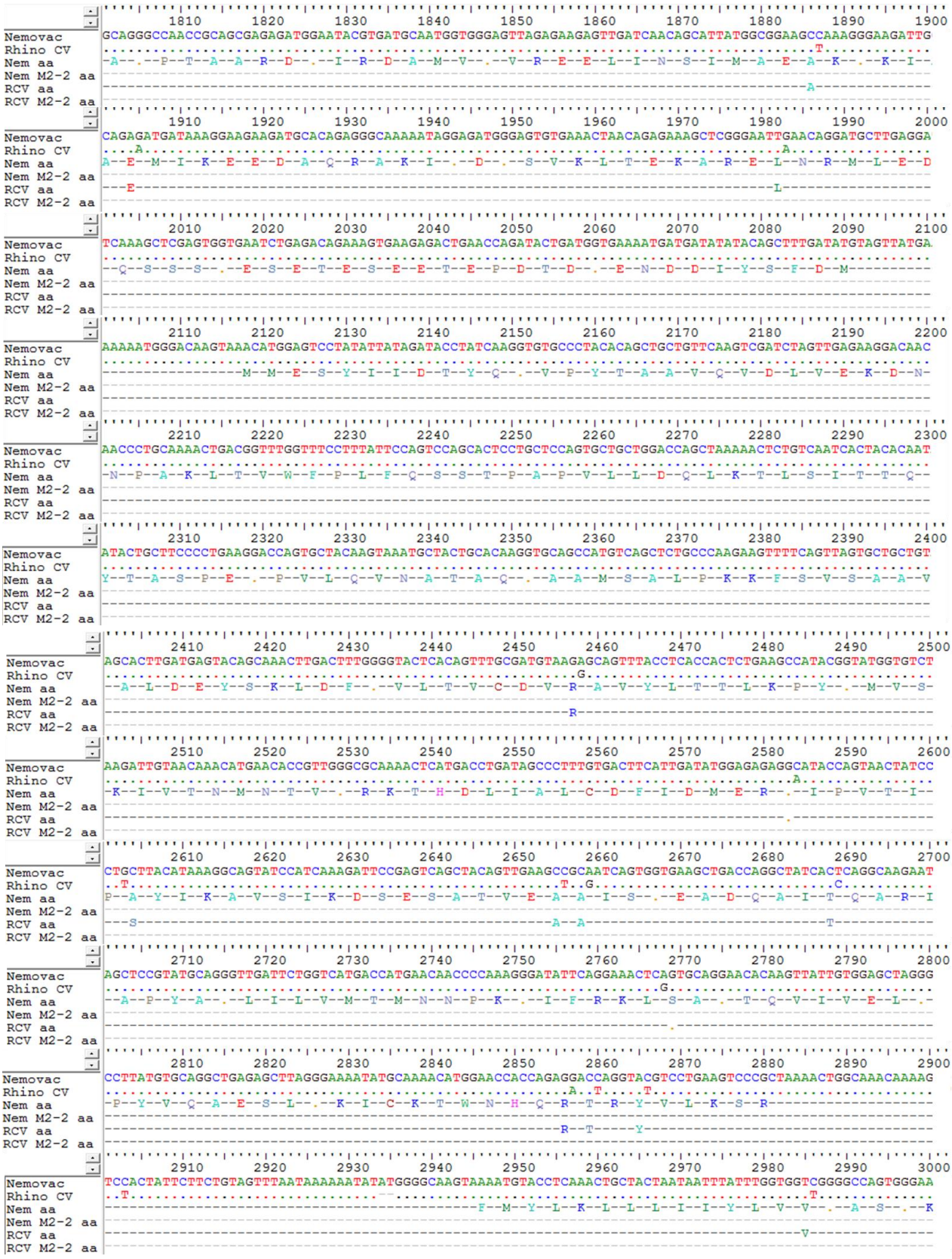
Rhino CV
Nem aa
Nem M2-2 aa
RCV aa
RCV M2-2 aa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Nemovac
Rhino CV
Nem aa
Nem M2-2 aa
RCV aa
RCV M2-2 aa
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2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
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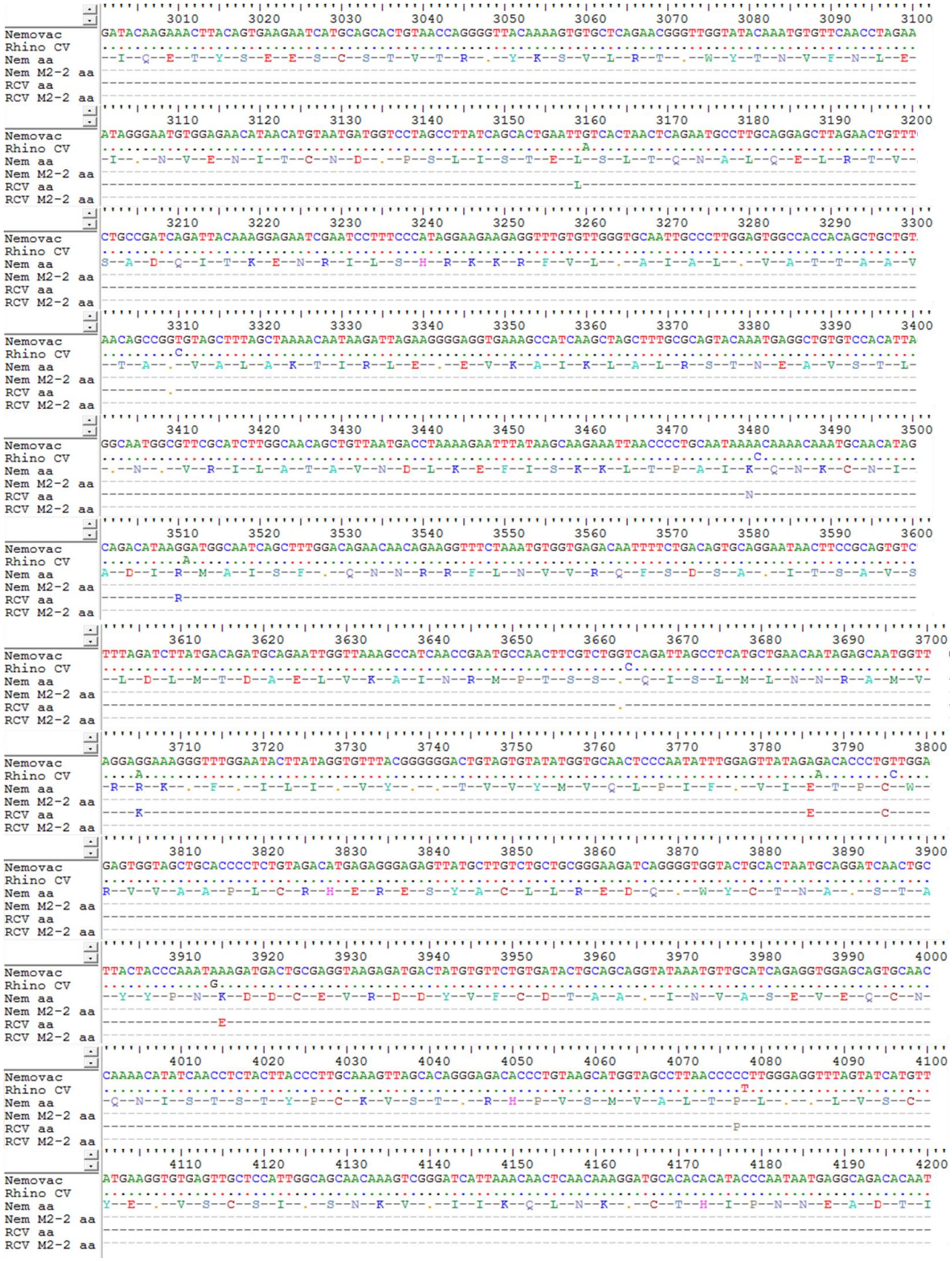
Nemovac
Rhino CV
Nem aa
Nem M2-2 aa
RCV aa
RCV M2-2 aa


4210 4220 4230 4240 4250 4260 4270 4280 4290 4300
Nemovac AACTATAGACAACACAATTTATCAACTCAGCAAGGTGGTGGGTGAGCAAAGGACCATCAAAGGAGCTCCGGTGGTAAATAATTTCAACCCTCTACTGTTC
Rhino CV .....T.....
Nem aa --T--I--D--N--T--I--Y--Q--L--S--K--V--V--.--E--Q--R--T--I--K--.--A--P--V--V--N--N--F--N--P--L--L--F-
Nem M2-2 aa
RCV aa --------D-----
RCV M2-2 aa
4310 4320 4330 4340 4350 4360 4370 4380 4390 4400
Nemovac CCTGAGGATCAATTTAACGTAGCCTTAGATCAAGTATTTGAGAGTGTTGATAAATCAAAAGACCTGATTGACAAGTCTAATGACTTACTGGACATAGAGG
Rhino CV .....A.
Nem aa -P--E--D--Q--F--N--V--A--L--D--Q--V--F--E--S--V--D--K--S--K--D--L--I--D--K--S--N--D--L--L--D--I--E--
Nem M2-2 aa
RCV aa ----------E--
RCV M2-2 aa
4410 4420 4430 4440 4450 4460 4470 4480 4490 4500
Nemovac TTAAGAGTAATATAGGTGCTGCATTAGCCATCACAATTTTAGCAGTGCTTAGCATGTCAATCATAGTGGGCATAGCTTACTATGTGGTTAAAAAGAGGAA
Rhino CV .....T.....
Nem aa V--K--S--N--I--.--A--A--L--A--I--T--I--L--A--V--L--S--M--S--I--I--V--.--I--A--Y--Y--V--V--K--K--R--K
Nem M2-2 aa
RCV aa ----------V-----
RCV M2-2 aa
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Nemovac AGCCAAAGCATCCAATGGATATCCTAAAACAACAGGGCAAAGCAACATGGGCTACATCAGTTGAGTTACATAAAAAGTGGGACAAGTAAAGATGTCCAGA
Rhino CV .....C.....
Nem aa --A--K--A--S--N--.--Y--P--K--T--T--.--Q--S--N--M--.--Y--I--S----------M-2-M--S--R-
Nem M2-2 aa
RCV aa ----------P-----
RCV M2-2 aa
4610 4620 4630 4640 4650 4660 4670 4680 4690 4700
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Rhino CV .....
Nem aa -R--N--P--C--R--Y--E--T--R--.--K--C--N--R--.--S--S--C--T--F--N--H--N--Y--W--S--W--P--D--H--V--L--L--
Nem M2-2 aa
RCV aa
RCV M2-2 aa
4710 4720 4730 4740 4750 4760 4770 4780 4790 4800
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Rhino CV .....
Nem aa V--R--A--N--Y--M--L--N--Q--L--V--R--N--T--D--R--T--D--.--L--S--L--I--S--.--A--.--R--E--D--R--T--Q--D
Nem M2-2 aa
RCV aa
RCV M2-2 aa
4810 4820 4830 4840 4850 4860 4870 4880 4890 4900
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Rhino CV .....A.....
Nem aa --F--V--L--.--S--A--N--V--V--Q--N--Y--I--E--.--N--A--T--I--T--K--S--A--A--C--Y--S--L--Y--N--I--I--K-
Nem M2-2 aa
RCV aa ----------A-----
RCV M2-2 aa
4910 4920 4930 4940 4950 4960 4970 4980 4990 5000
Nemovac CAACTTCAAGAGAATGATGTGAAATCTGCACGAGACCTGATGGTAGATGACCCCAAGCATGTTGCCCTGCATAACCTTGTCTTGTCCTATATAGACATGA
Rhino CV .....
Nem aa -Q--L--Q--E--N--D--V--K--S--A--R--D--L--M--V--D--D--P--K--H--V--A--L--H--N--L--V--L--S--Y--I--D--M--
Nem M2-2 aa
RCV aa
RCV M2-2 aa
5010 5020 5030 5040 5050 5060 5070 5080 5090 5100
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Rhino CV .....
Nem aa S--K--N--P--A--N--L--I--N--S--L--K--R--L--P--K--E--K--L--K--K--L--A--K--I--I--I--Q--L--S--A--.--S--E
Nem M2-2 aa
RCV aa
RCV M2-2 aa
5110 5120 5130 5140 5150 5160 5170 5180 5190 5200
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Rhino CV .....A....A.....A.....C.....
Nem aa --.--E--N--A--N--S--N--T--L--Q--K--.--D--.--S--N----------
Nem M2-2 aa ---M2.2--M--P--I--V--I--P--C--K--R--V--T--A--V--I--R--C--N--T--L--.--V--C--L--F--K--R--T--Y--E--H--N
RCV aa ----------D-----S----------
RCV M2-2 aa ----------M--T----------K----------S----------
5210 5220 5230 5240 5250 5260 5270 5280 5290 5300
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Rhino CV CATCATTAACCTTGGTGATCTGATAGAAGAAGTAGCGAGGATGATTATTATAGATCACATAAATAGAAAACAATGCAATGAATGTAGAAAAGATTTT--G
Nem aa ----------
Nem M2-2 aa --I--I--N--L--G--D--L--I--E--E--V--A--R--M--I--I--I--I--D--H--I--N--R--K--Q--C--N--E--C--R--K--D--F--L-
RCV aa ----------
RCV M2-2 aa ----------R----------
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Rhino CV AATTTGTAGCGGTTTACACATCTTACACTTAGTTATATAAAAA-CAATTGAGCACCCCCCCCGAAAAAAGATGGGGCAAGTCAAGATGACCTCCACTGTC
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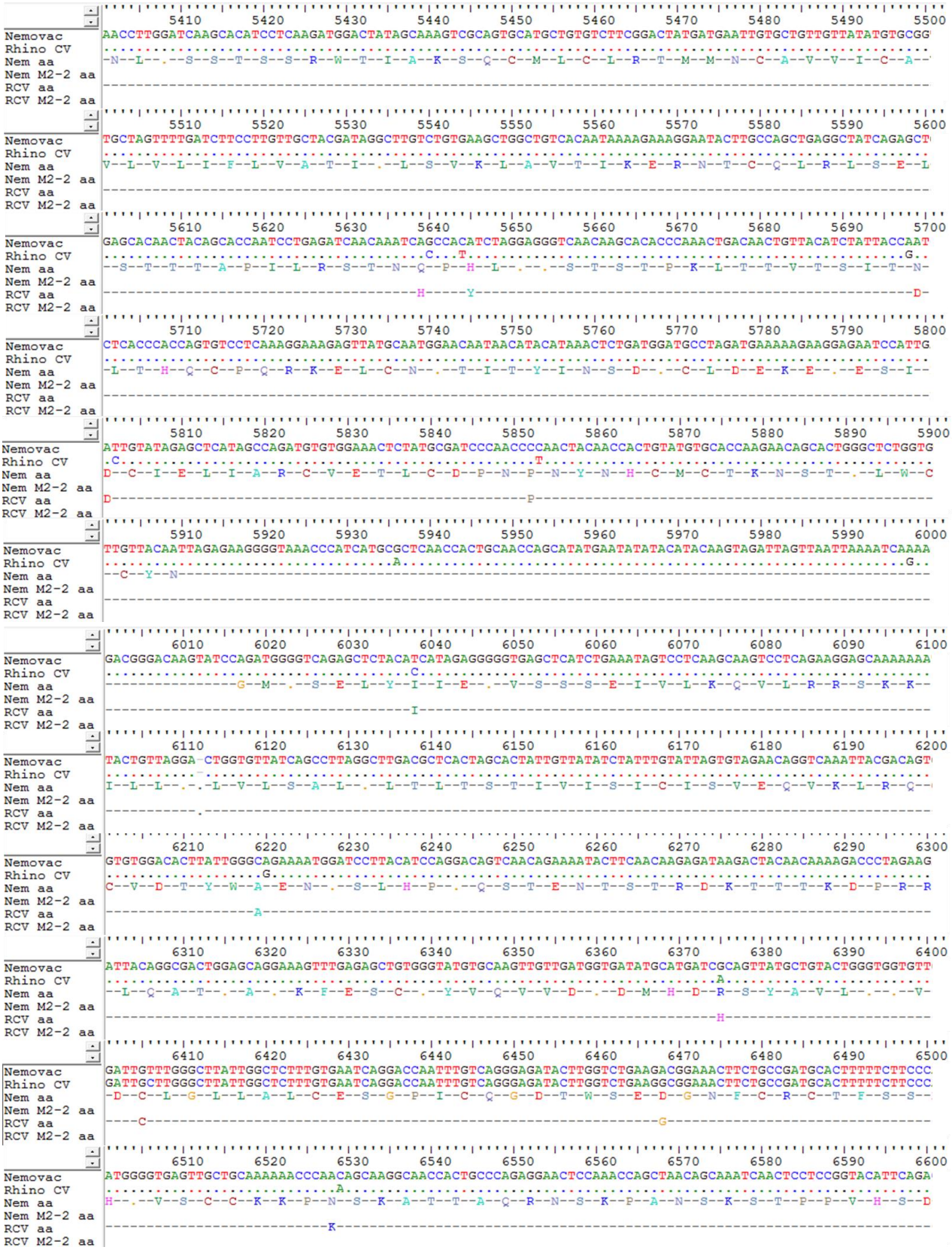
Nem M2-2 aa -N--L--
RCV aa ----------K----------
RCV M2-2 aa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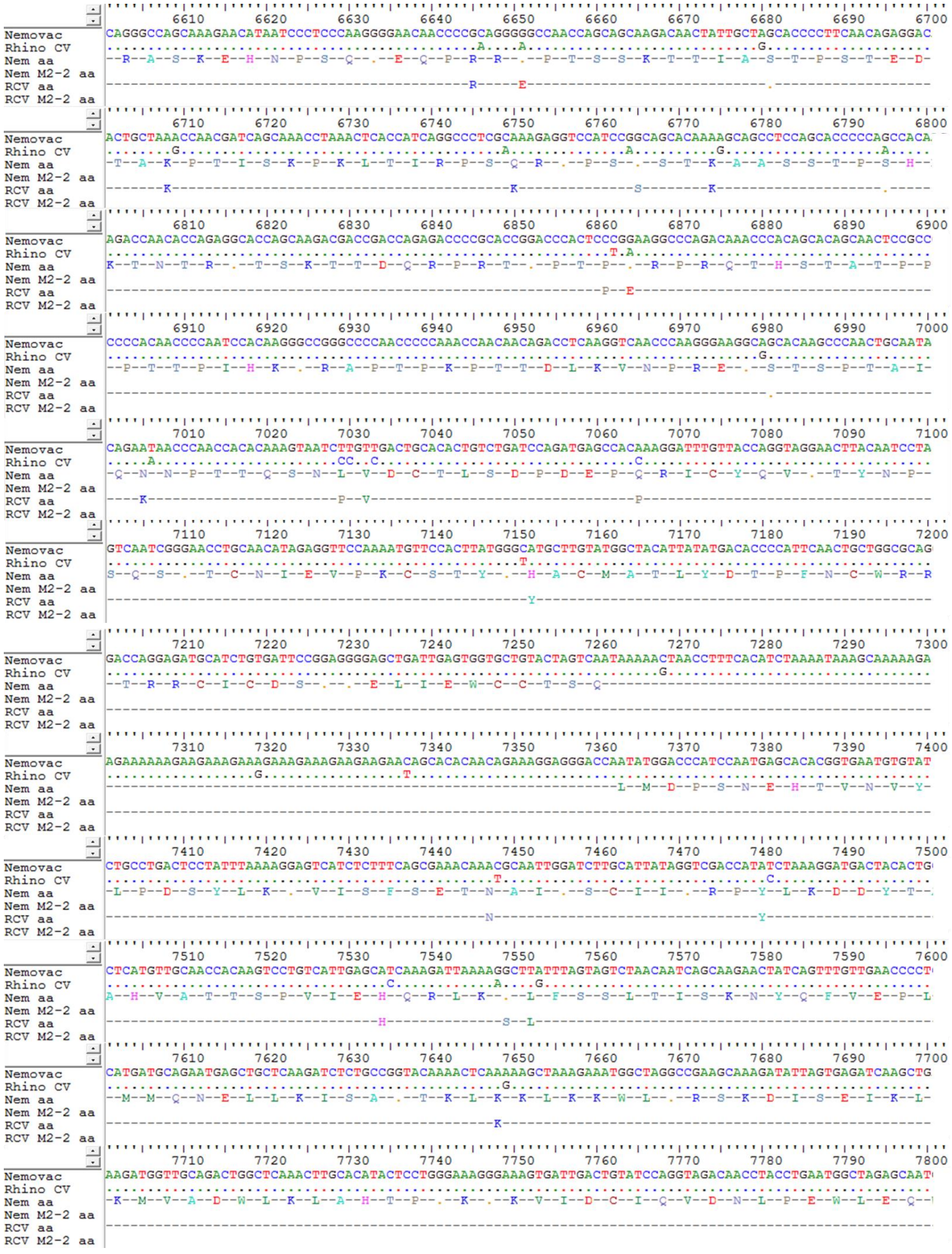
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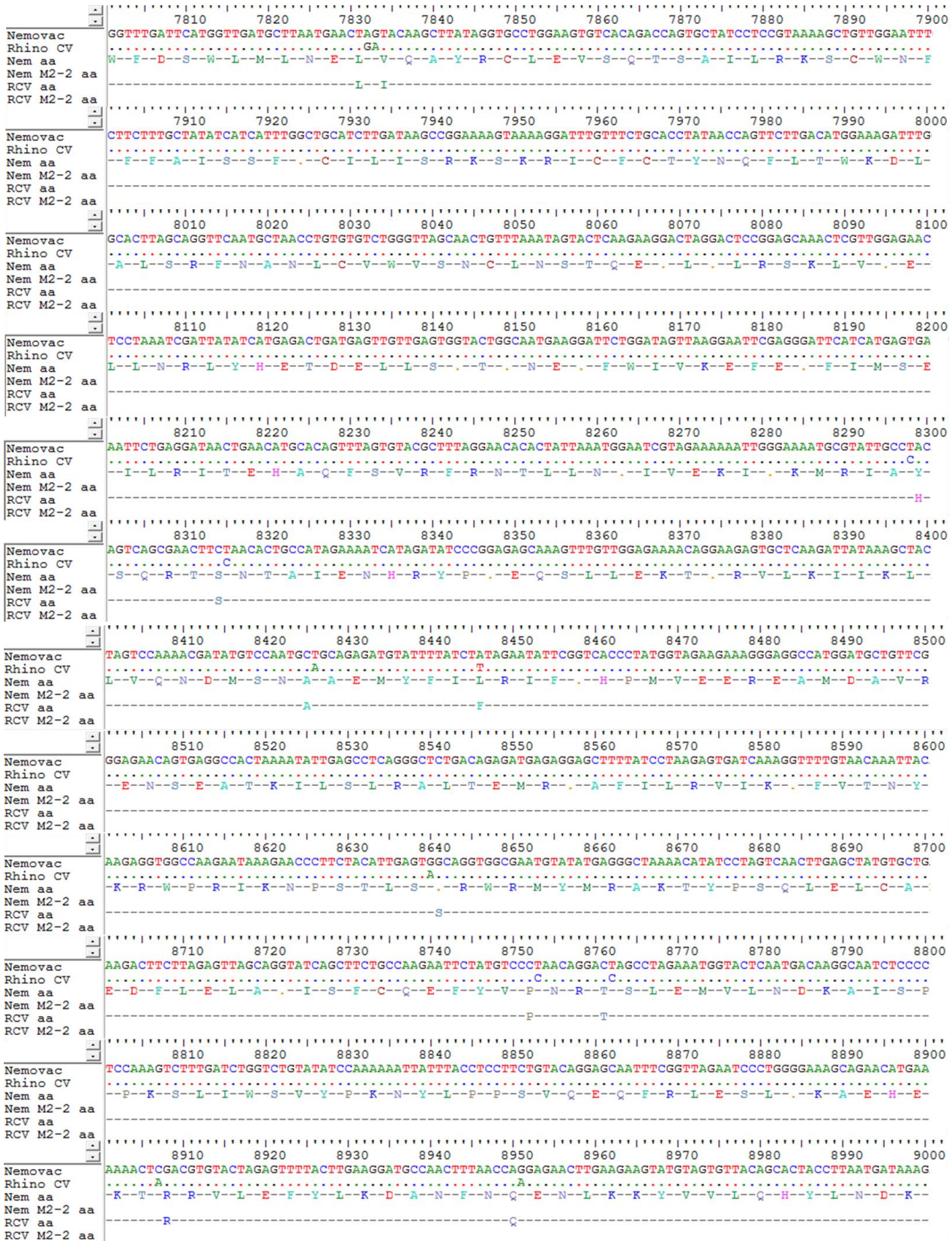
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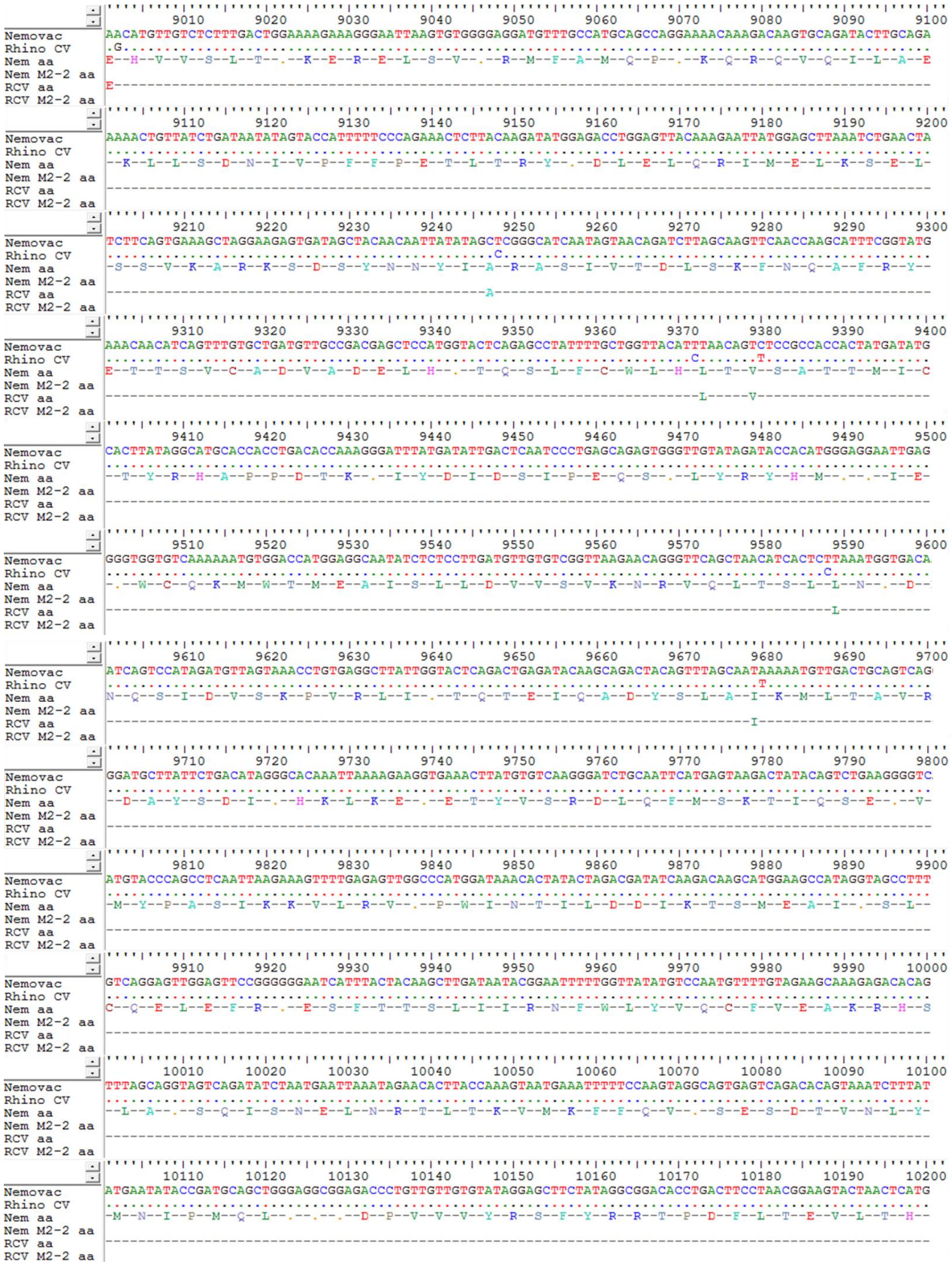
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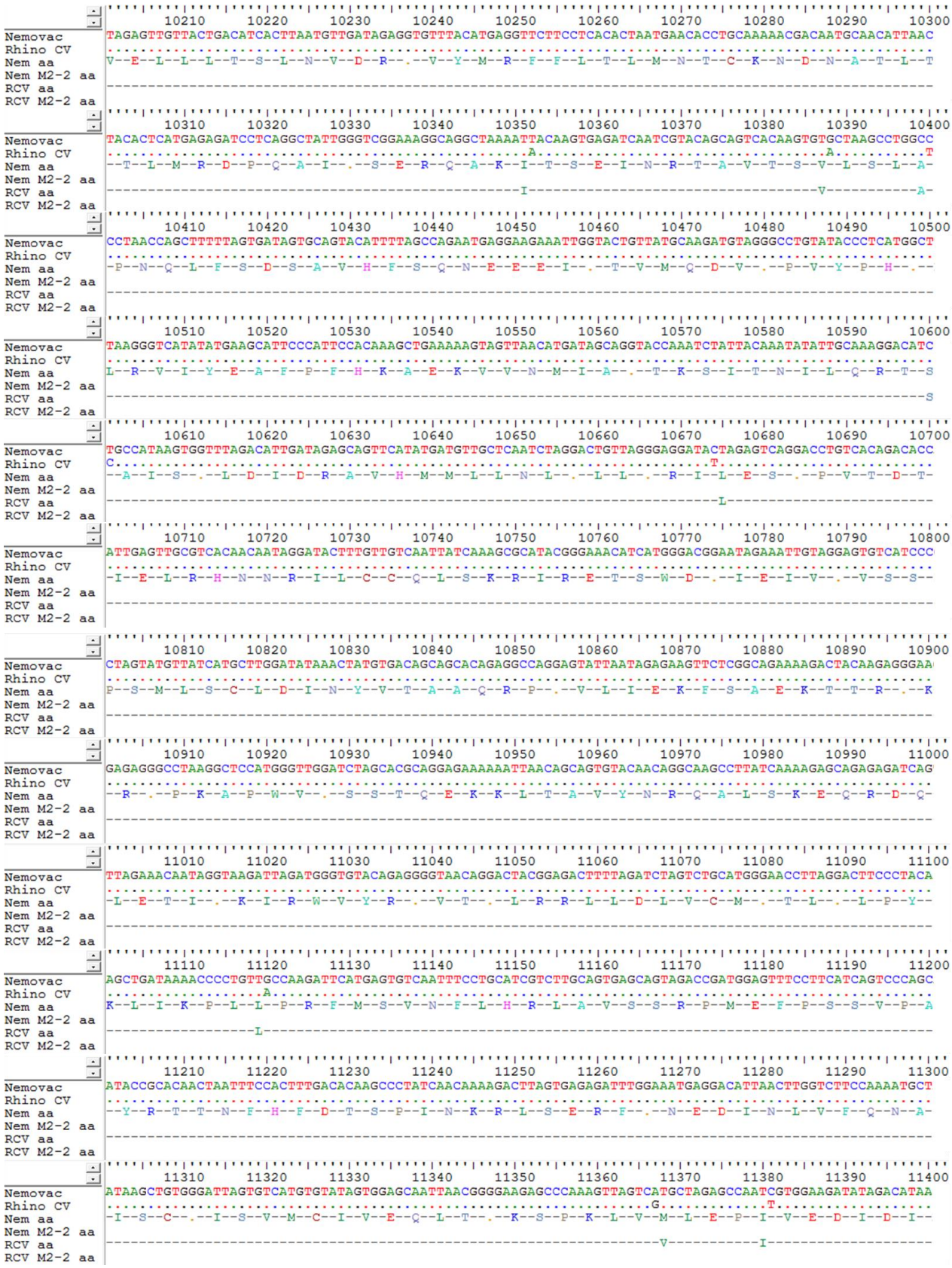
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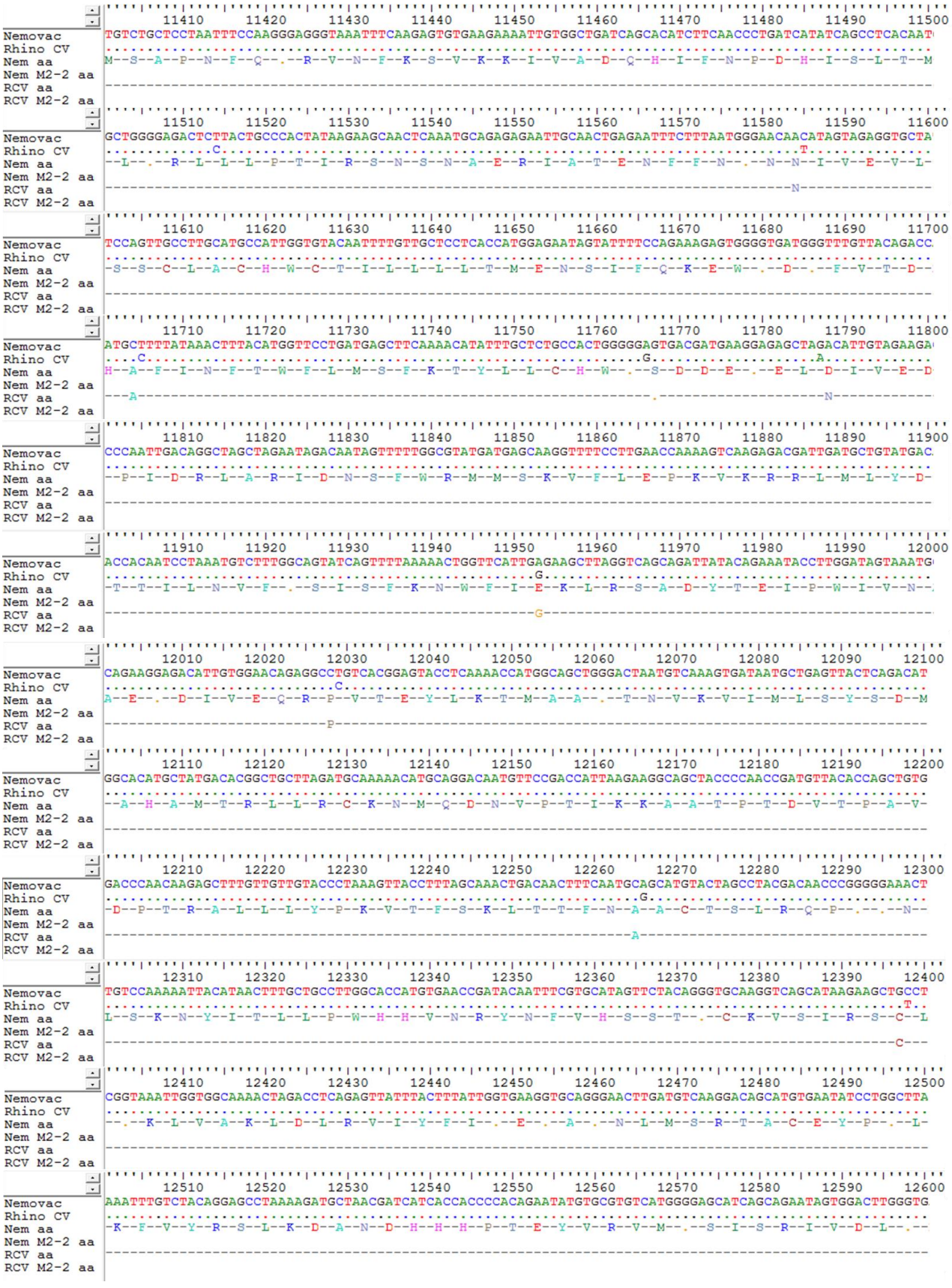
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Nem M2-2 aa
RCV aa
RCV M2-2 aa
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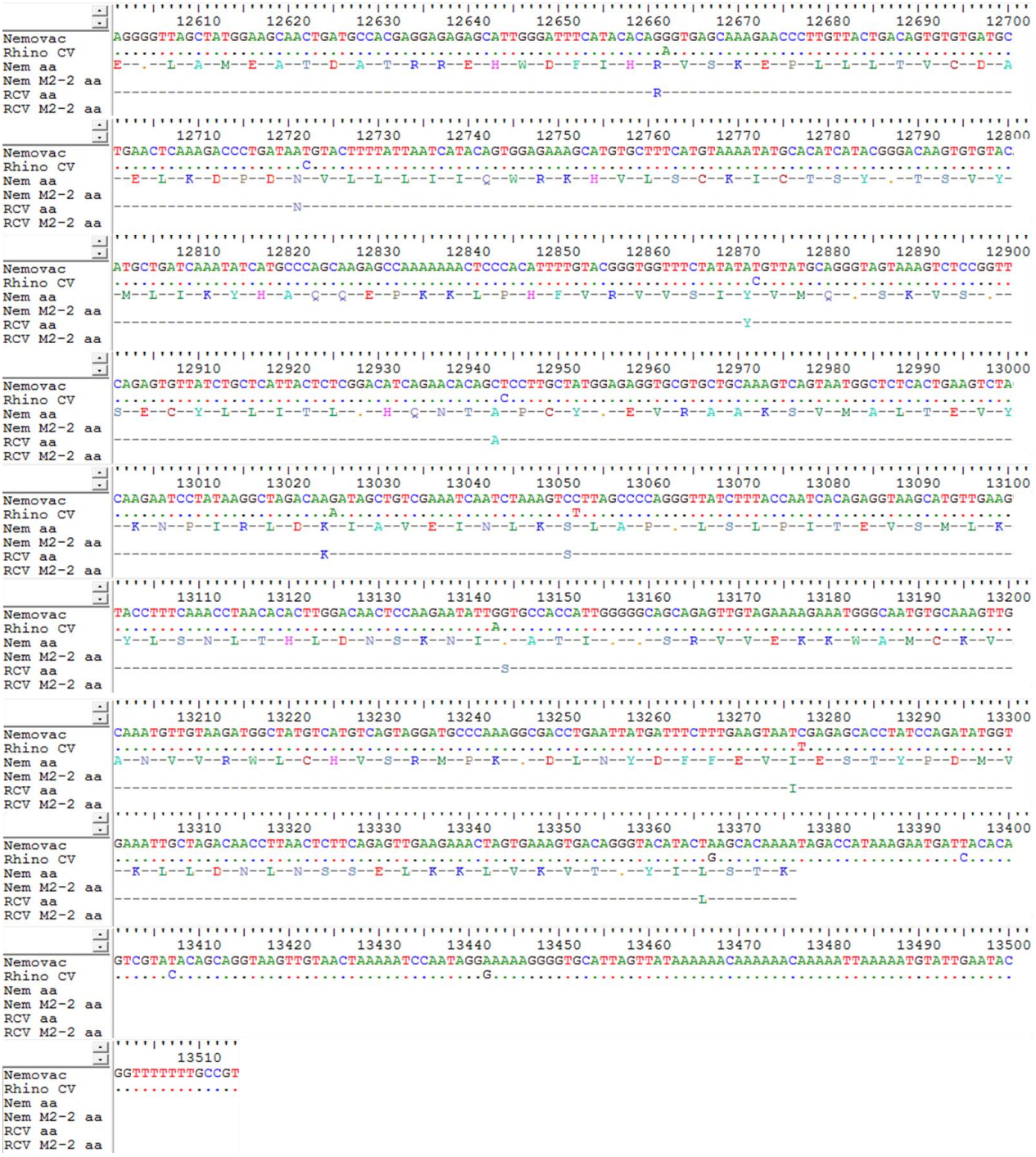

Figure 6.3 Nucleotide and aminoacid alignment of Nemovac and RhinoCV.

6.3.2 Chimera AMPV-B construction

Two amplicons covering the F, M2, SH and G genes were generated and then used in two consecutive SDM steps. After the two SDM steps, only one plasmid showed the correct RE pattern. The sequence analysis of the plasmid confirmed that the F, M2, SH, and G gene were fully converted to Nemovac sequence. The plasmid was named cvB (chimera vector subtype B).

6.3.3 Exogenous genes addition

The cloning cassette was added at the G-L intergenic region. The N and the S1 amplicons obtained by RT-PCR were cloned into the cassette in two separate reactions. Four FL plasmids containing the N gene were positive both by PCR and RE screening, while 3 plasmids were positive for the S1 gene insertion. The sequence analysis of the inserted genes did not show any mutations. Interleukin-18 was amplified by PCR and then cloned. Three plasmids containing the N and the IL-18 genes and 5 plasmids containing the S1 and the IL-18 genes were generated. Sequencing analyses confirmed that no mutations were present in any of the exogenous genes.

		Nucleotide changes		Amino acid chamges	
Gene	**Position**	**Nemovac**	**RhinoCV**	**Nemovac**	**RhinoCV**
N	375	A	G	Glutamine	Arginine
	905	A	G	Lysine	Glutamic acid
M	2603	C	T	Histidine	Tyrosine
	2768	A	G	Serine	Glycine
F	3481	A	C	Leucine	Asparagine
	3705	G	A	Arginine	Leucine
	3914	A	G	Lysine	Glutamic acid
	4443	C	T	Alanine	Valine
	4511	T	C	Serine	Proline
M2:1	5136	G	A	Glycine	Aspartic acid
	5141	G	A	Glycine	Serine
M2:2	5136	G	A	Valine	Methionine
	5152	G	A	Serine	Lysine
	5164	T	C	Leucine	Serine
SH	5383	G	A	Glutamic acid	Lysine
	5640	G	C	Glutamin	Histidine
	5644	C	T	Histidine	Tyrosine
	5699	A	G	Asparagine	Glutamic acid
G	6375	G	A	Arginine	Histidine
	6468	A	G	Aspartic acid	Glycine
	6529	C	A	Asparagine	Lysine
	6651	G	A	Glycine	Glutamic acid
	6680	A	G	Serine	Glycine
	6749	C	A	Glutamine	Lysine
	6764	G	A	Glycine	Serine
	6795	G	A	Serine	Aspartic acid
	6864	G	A	Glycine	Glutamic acid
	7006	T	A	Aspartic acid	Lysine
	7029/7030	TT	CC	Leucine	Proline
	7065	A	C	Glutamine	Proline
	7151	C	T	Histidine	Tyrosine
L	7548	G	A	Glycine	Serine
	7833	G	A	Valine	Isoleucine
	8298	T	C	Tyrosine	Histidine
	8447	A	T	Leucine	Phenylalanine
	8640	G	A	Glycine	Serine
	11367	A	G	Methionine	Valine
	11766	A	G	Serine	Glycine
	11787	G	A	Aspartic acid	Asparagine
	11953	A	G	Glutamic acid	Glycine
	13143	G	A	Glycine	Serine

Table 6.3 List of the coding changes detected after comparison of nucleotide and aminocid sequence of Nemovac and RhinoCV. The highlighted changes were modified by SDM.

6.3.4 Recovery of recombinant viruses

Not all the plasmids transfected into Vero cells were recovered. The presence of cytopathic effect (CPE) on the Vero cell monolayers was observed for cvB, for one of the $cB_{S1\ G\text{-}L}$ constructs, for one of the $cB_{N\ G\text{-}L}$ constructs and for one of the $cB_{S1+IL\text{-}18\ G\text{-}L}$ constructs. No CPE was observed in any of the cell sheets transfected with construct $cB_{N+IL\text{-}18\ G\text{-}L}$. RT-PCR targeting the AMPV viral mRNA detected the viral mRNA only in monolayers showing CPE. After 3 passages on Vero cells, all the recombinant viruses reached a titre sufficient for a protection study. Different titres were obtained for the recombinants; the highest titre was observed for $cB_{N\ G\text{-}L}$ virus, reaching 6.5 log/ml. The sequencing of the insert genes showed gene sequences to be correct.

Clone name	**Insert gene**	**No. Plasmids**	**No. Rescued**	**Titre**
cvB	none	1	1	5.5 log_{10}/ml
$cB_{N\ G\text{-}L}$	N	4	1	6.5 log_{10}/ml
$cB_{S1\ G\text{-}L}$	S1	3	1	5.6 log_{10}/ml
$cB_{N+IL\text{-}18\ G\text{-}L}$	N and IL-18	3	None	n.d.
$cB_{S1+IL\text{-}18\ G\text{-}L}$	S1 and IL-18	5	1	5.5 log_{10}/ml

Tabella 6.4 Summary of the constructed recombinant cloned FL cDNAs, of the recombinant viruses rescued and their titre on VERO cell

6.3.5 Sequences comparison of two $cB_{N\ G\text{-}L}$ plasmids

The full genome sequences of two plasmids containing the N gene ($cB_{N\ G\text{-}L}$) were analysed. No nucleotide changes were observed between plasmid 6_{29}, successfully rescued, and plasmid 6_{27}, not rescued.

6.3.6 Exogenous genes transcription and expression

RT-PCR targeting the mRNA demonstrated that the recombinant viruses actively transcribed the exogenous genes. Immunofluorescence confirmed that the exogenous proteins were expressed by all the recombinants (see pictures 6.4).

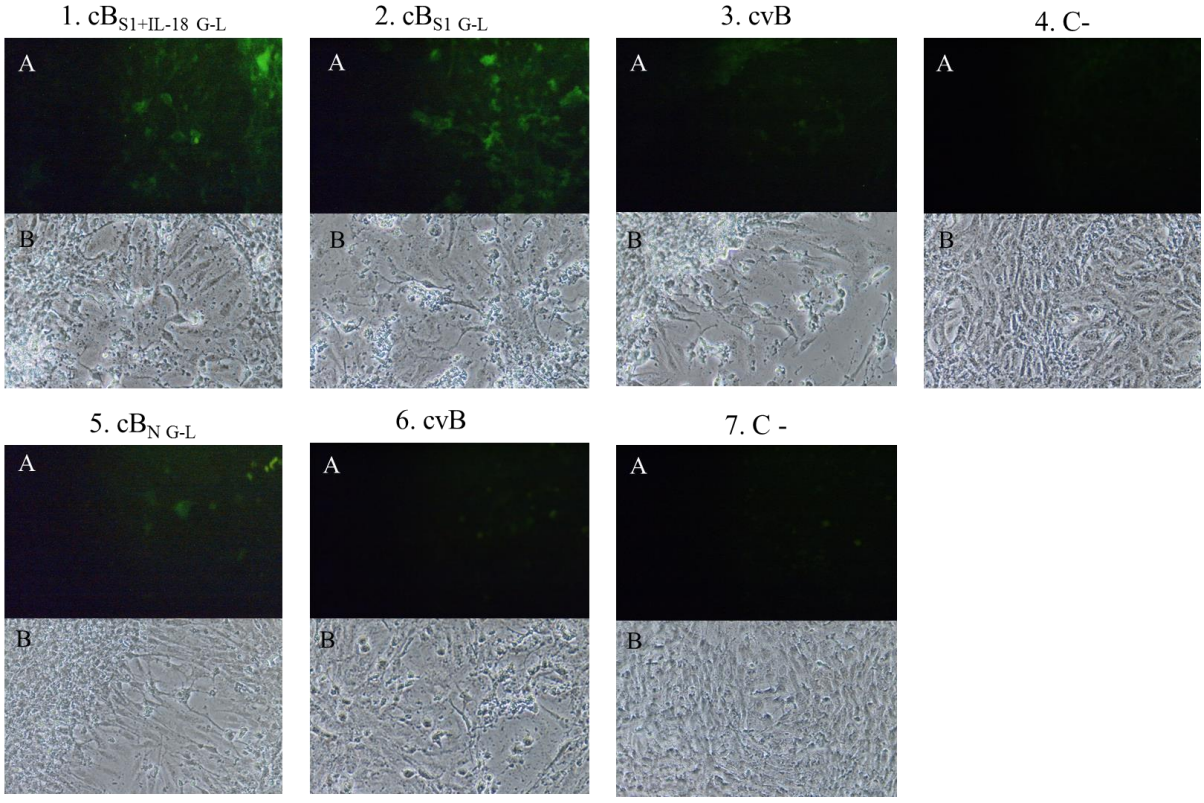

Figure 6.4 Microscopy of VERO cells infected with 3 AMPV-B/IBV recombinants and negative control. A: immunofluorescence using FITCH specific antibodies. B: white light illumination. Viruses 1 to 4 used IBV polyclonal chicken serum, viruses 5 to 7 used N monoclonal serum.

6.3.7 Recombinants replication *in vivo*

All the recombinants tested replicated in the trachea of the birds. The recombinant showing the higher replication was the cB$_{S1\ G-L}$. Construct cB$_{N\ G-L}$ replicated better when administered to the birds at the lower dose (Table 6.5).

6.3.8 Tracheal motility following challenge

On 5 and 6 days post challenge (d.p.c.), recovery of the cilial beating was observed in all the groups vaccinated with the recombinants. The protection varied among the different recombinants and $cB_{N\ G\text{-}L}$ group showed the best protection (Table 6.5). Stronger cilial recovery was observed in the birds vaccinated with $cB_{S1+IL\text{-}18\ G\text{-}L}$ compared to $cB_{S1\ G\text{-}L}$ (Table 6.5). Nevertheless, none of the recombinants showed the cilial recovery required by the European Pharmacopoeia.

Birds vaccinated with the commercially available Mass vaccine showed full protection. Unvaccinated/challenged birds showed no cilial motility, while unvaccinated/unchallenged birds showed full motility (Table 6.5). Low recovery was observed also in birds vaccinated with cvB (Table 6.5).

Groups	**Real time (5 d.p.v.)**		**%TOC beating**	**50% beating rings**
	Positive	**I.D. (mean)**		
cvB	8/10	1000	20%	2%
$cB_{N\ G\text{-}L}$ (low dose)	6/10	100	86%	14%
$cB_{N\ G\text{-}L}$ (high dose)	10/10	1000	70%	13%
$cB_{S1\ G\text{-}L}$	10/10	1000	42%	5%
$cB_{S1+IL\text{-}18\ G\text{-}L}$	7/10	100	48%	5%
Mass	n.d.	n.d.	100%	100
Unvacc/challenged	0/5	0	0%	0%
Unvacc/unchallenged	0/5	0	100%	100

Tabella 6.5 Effects of vaccination with three subtype B recombinants on virus replication, antibody response to AMPV and % TOC beating after challenge with IBV.

6.4 DISCUSSION

The comparison of the full genome sequence of RhinoCV (Intervet) and Nemovac (Merial) revealed that most coding changes (27) were located in the genes coding

for the proteins involved in the fusion and release of the virions from the host cell surface (Fusion (F), Small Hydrophobic (SH) and the Attachment (G) proteins). A plasmid characterized by a hybrid sequence of RhinoCV (N, P, M and L genes) and Nemovac (F, M2, SH and G genes) was generated and rescued. A comparison of the viral titres detected in the trachea of birds vaccinated respectively with the chimera virus and with RhinoCV revealed that the chimera titres was 100 times higher, suggesting that the chimera virus has a better potential to deliver foreign genes.

AMPV-B/IBV stable recombinants based on the hybrid sequence were generated, inoculated into SPF chickens and challenged against a virulent Mass IBV strain: cilial recovery was observed in all the groups. The cilial recovery was markedly higher than that observed in chapter 5 for RhinoCV recombinants and the comparison of the virus titres detected in the trachea of the birds revealed that the chimera recombinants reached titres 1000 times higher than the previous subtype B recombinants. These data confirm that high replication in the respiratory tract doesn't imply the induction of protective immunity. Nevertheless the pharmacopoeia requirements were not matched. It's possible that despite the high replication, the expression of the exogenous genes wasn't enough to stimulate a strong immune response in the host. The transcription mechanism of AMPV involves a gradually decrease in the mRNA production moving from the 3' end to the 5' end (Dimmock et al., 2007): inserting the exogenous gene into an intergenic region closer to the leader will increase the exogenous protein expression. Nevertheless, the site of insertion must be chosen very carefully, as in the previous study on subtype A recombinants indicated that the insertion of an exogenous gene too close to the 3' end affected the virus viability (Falchieri et al., 2013).

Differences in term of protection between the constructs were observed, with the highest cilial recovery detected in the birds vaccinated with the recombinants

expressing the N protein, in accordance with a study carried on using subtype A recombinants (Falchieri et al., 2013) but in contrast to a previous study conducted using subtype B recombinants (chapter 5), where no difference in terms of induction of protection was observed. To explain these contrasting results, it was previously supposed that that the low level of replication of the previous subtype B recombinants might have prevent a sufficient IBV proteins expression to stimulate the immune response in the host and the data obtained in the present study seems to confirm this hypothesis.

For the first time a subtype B recombinant expressing more than one exogenous gene was rescued. A major cilial recovery was observed for the birds vaccinated with recombinant expressing both S1 and IL-18, when compare to those vaccinated with recombinant expressing only the S1 protein, confirming that the co-expression of IL-18 enhances the vaccine efficacy. The recovery of virus expressing N and IL-18 proved to be impossible. The presence of these two exogenous genes placed in the same intergenic region might have prevented the transcription of the downstream gene, probably as a result of an interaction between the two exogenous sequences that led to the formation of RNA secondary structures. It would be interesting to move the N gene to another intergenic region and then attempt the rescue of the virus containing both genes. Considering that the N protein proved able to induce better protection than the S1 and that IL-18 confirmed its ability to enhance vaccine efficacy, it's likely that a recombinant expressing N and IL-18 proteins might represent a step ahead in the development of effective AMPV-B/IBV recombinant vaccines. Despite the conflicting data, the ability of subtype B to accept and express two foreign genes could also be used to produce recombinants expressing both N and S1. In the study on subtype A recombinants, those expressing the two IBV proteins conferred the best protection (Falchieri et al., 2013).

For each construct, several plasmids were obtained, confirming that subtype B could be readily manipulated and suggesting that it would be possible to generate a large range of viruses for testing as candidate vaccines in protection studies. Nevertheless only a few viruses were rescued. In a previous study investigating AMPV adaptation on Vero cells it was shown that a single non conservative mutation in the M2:2 protein could prevent the virus being rescued (Clubbe et al., 2011). It was therefore it was supposed that the low successful rate could be due to mutations introduced into the AMPV genome during one of the steps of the development of the plasmids. The comparison of the full genome sequence of two plasmids of construct $cB_{N\ G\text{-}L,}$ one rescued and one not rescued, did not show any nucleotide changes between them, seemingly disproving the mutations theory. This suggests that virus recovery is not totally reliable and that small changes in conditions to the reverse genetics system might have a large effect on outcomes.

The chimera recombinant expressing the N gene showed an unexpected behaviour *in vitro* in reaching titres higher than any other recombinants and even higher than virus rescued from the unmodified vector. It may be possible that the IBV N gene enhanced the replication *in vitro*, even if a similar behaviour had not been observed in either the previous study on subtype B recombinants, nor in the subtype A study (Falchieri et al., 2013). If confirmed, the ability of the N gene to enhance the replication *in vitro* could be very useful at the production stage of the vaccine, as if sufficient titre per cell culture cannot be achieved, promising vaccines could be otherwise rejected due to required inoculation doses being unattainable.

A group of birds was vaccinated with a 10 times higher dose of recombinant expressing the N protein. When compared to birds vaccinated with the usual dose, both the level of replication in the trachea and induced protection were reduced. Counterintuitively, the data might indicate that the vaccine dose is crucial for

efficient vaccination and that a higher viral dose might negatively affect the replication, and lead to a reduction in protection.

In conclusion, while the chimera recombinants did not confer full protection, higher replication in the trachea and increased cilial recovery indicate that a step forward has been made in the development of effective recombinant AMPV-B/IBV vaccines. The recombinants confirm their ability to be actively transcribed and express exogenous genes. Furthermore the N protein has been confirmed as the major antigenic protein of IBV, IL-18 confirmed its ability to enhance the effectiveness of vaccines, subtype B was shown able to incorporate more than one exogenous gene and the failure in the recovery of virus expressing N and IL-18 proteins suggested that the cloning of two foreign genes in the same intergenic region might affect the virus viability. As future developments, the generation of subtype B constructs containing IBV and IL-18 genes at two different intergenic region, as well as constructs containing both S1 and N, would be of interest.

7. INVESTIGATING THE ABILITY OF SUBTYPE B AVIAN METAPNEUMOVIRUS TO ACCEPT EXOGENOUS GENES AT MULTIPLE INTERGENIC POSITIONS

7.1 INTRODUCTION

In the two previous chapters, the development of two sets of avian metapneumovirus (AMPV) subtype B recombinant vaccines expressing infectious bronchitis virus (IBV) immunogenic proteins was described. None were able to induce enough protection at challenge to meet the pharmacopoeia requirements. The first set of recombinants used RhinoCV (Intervet) vaccine as vector to express N or S1 protein of IBV. Protection induced by the recombinants was challenge *in vivo* using an IBV homologous strain. The replication was poor replication in the trachea of the birds and little conferred protection. To increase the protection, a chimera vector with a hybrid RhinoCV/Nemovac (Merial) sequence was generated. Chimera recombinant viruses replicated to high titres in the trachea of the birds, but did not confer full protection at the challenge. It was supposed that the partial protection might be due to poor expression of exogenous proteins and that increasing the exogenous protein expression will improve the protection.

Using the subtype A RG system, Falchieri et al. (2013) introduced a reporter gene at each AMPV intergenic region and observed a decrease in the expression of the exogenous protein moving from the leader to the trailer. The maximum exogenous protein expression was observed at the N-P junction. These data are in accordance with a transcription mechanism for AMPV resulting from the viral polymerase only joining the genome at the viral leader and the polymerase being able to detach from

the genome at any stage before the trailer is reached (Dimmock et al., 2007). The same study revealed that the viability of the virus could be influence by the position of the reporter gene. High virus titres were obtained only when the insert gene was placed at the M-F junction or at downstream junctions, suggesting that in subtype A the M-F junction represent the best compromise between exogenous gene expression and virus viability (Falchieri et al., 2013). In the present study it was assumed that the same applies to subtype B. The N gene of a Massachusetts (Mass) IBV strain was therefore cloned into the M-F junction of the chimera plasmid.

For subtype A, AMPV-A\IBV recombinants recombinant containing both the N and the S1 genes achieved the best protection after challenged with homologous IBV(Falchieri et al., 2013). IBV N protein is reported to stimulate a cell mediate immune response (CMI) (Seo et al., 1997) whereas the S1 protein is reported to stimulate antibody production (Cavanagh et a., 1986: Mockett et al., 1984). In the present study the N gene was cloned at the M-F intergenic region of two chimera plasmids containing respectively the solely S1 gene or both the S1 and the IL-18 genes at the G-L junction.

The Matrix (M) protein is one of the structural and surface proteins of IBV. To date there were no study showing this to be an immunogenic protein, but nevertheless its position on the viral surface suggest that the M protein should be further investigated. To study the role played by this protein and to potentially increase the ability of the recombinants to confer protective immunity, the M gene sequence was cloned into the G-L intergenic region of the chimera plasmid containing also the N gene; and in the M-F intergenic region of the chimera plasmid containing the S1 and the IL-18 genes.

All the constructs were transfected into Vero cells. Several virus rescue attempts were performed.

7.2 MATERIALS AND METHODS

7.2.1 Plasmids preparation

7.2.1.1 N gene cloning in the M-F intergenic region of the chimera vector (cvB).

An XhoI restriction endonuclease (RE) site was added by site directed mutagenesis (SDM) (see methods chapter (mc) 3.7) into the M–F intergenic region of a plasmid containing an avian metapneumovirus subtype B chimera genome copy, based on RhinoCV (Intervet) and Nemovac (Merial) vaccines. As described in paragraph 5.2.1, the cloning cassette contained a complimentary DNA copy of a transcriptional start (GGGACAAGT), a Sal I restriction endonuclease site (GTCGAC) and a complimentary DNA copy of a transcriptional stop (AGTCAATAAAAAA). The cassette was ligated (mc 3.6) into the XhoI site, the plasmids transformed and liquid cultured (mc 3.8), purified (mc 3.1), and screened by PCR (mc 3.3) and RE (mc 3.9). Correct plasmids were selected by sequencing (mc 3.5) of the cassette.

Infectious bronchitis virus (IBV) RNA was extracted from a Massachusetts (Mass) field strain using Qiamp Viral RNA minikit (mc 3.1) and reverse transcribed (mc 3.2). The cDNA was amplified (m 3.3) using primers introducing an XhoI site at the sequence ends. The amplicons were ligated (mc 3.6) into the cassette. The plasmid were transformed on STB12 cells (mc 3.8), screened by PCR (mc 3.3) to check for the correct orientation of the N gene, liquid cultured (mc 3.8), purified (mc 3.1) and checked for integrity by RE (mc 3.9). The insert gene was amplified (mc 3.3) and the sequences generated analysed (mc 3.5).

7.2.1.2 N gene cloning in the M-F intergenic region of plasmids containing the S1 and IL-18 genes in the G-L intergenic region ($cB_{S1+IL\text{-}18\ G\text{-}L}$)

A high fidelity PCR (mc 3.3), using primers matching either side of the cassette, was performed on the plasmid containing the cassette in the M-F intergenic region. The amplicons generated were used as megaprimers in an SDM (mc 3.7) having as template plasmids containing the S1 and the IL-18 genes ($cB_{S1+IL\text{-}18\ G\text{-}L}$) in the G-L intergenic region. The N gene was ligated (mc 3.6) in the cassette, the plasmids transformed (mc 3.8), screened by PCR (mc 3.3), liquid cultured (mc 3.8) and checked for integrity with RE digestion (mc 3.9). Finally the N gene was amplified (mc 3.3) and sequenced (mc 3.5).

7.2.1.3 M gene cloning in the M-F integenic region of plasmids containing the S1 and IL-18 ($cB_{S1+IL\text{-}18\ G\text{-}L}$) genes in the G-L intergenic region

IBV RNA was extracted (mc 3.1) from a Mass field strain and reverse transcribed (mc 3.2). The cDNA was amplified (mc 3.3) using primers introducing XhoI site at the extremity of the M gene. The product generated was ligated (mc 3.6) into the cloning cassette at the M-F junction of the chimera plasmid also containing the S1 and IL-18 genes in the G-L intergenic region ($cB_{S1+IL\text{-}18\ G\text{-}L}$). After transformation (mc 3.8), the plasmid was screened by PCR (mc 3.3), liquid cultured (mc 3.8), purified (mc 3.1) and cut with RE (mc 3.9). The M genes of positive clones was amplified and sequenced.

7.2.1.4 M gene cloning in the G-L intergenic region of plasmids containing the N gene in the M-F intergenic region

A high fidelity PCR (mc 3.3), using primers matching either side of the cassette, was performed on the plasmid containing the cassette in the G-L intergenic region. The amplicons generated were used as SDM megraprimers to introduce the cassette at the G-L junction of the plasmid containing the N gene in the M-F intergenic region. The M gene was ligated (mc 3.6) into the cassette at the G-L junction, the plasmids transformed (mc 3.8), screened by PCR (mc 3.3), liquid cultured (mc 3.8), purified (mc 3.1) and cut with RE (mc 3.9). The M gene was amplified (mc 3.3) and sequenced (mc 3.5).

7.2.1.5 S1 gene cloning in the G-L intergenic region of plasmids containing the N gene in the M-F intergenic region

The S1 gene inserted in plasmid $cB_{S1\ G\text{-}L}$ was amplified using high fidelity PCR (mc 3.3). The amplicons generated were used as megaprimers in an SDM, having as template the plasmid containing the N gene at M-F junction. The SDM products were transformed (mc 3.8), screened by PCR (mc 3.3), liquid cultured (mc 3.8) and checked for integrity by RE digestion (mc 3.9). The S1 gene was amplified (mc 3.3) and sequenced (mc 3.5).

7.2.2 Recovery of recombinant viruses

The plasmids containing the modified sequence were transfected on Vero cells in the presence of subtype B support genes, following the protocol described in paragraph 4.6. The cell sheets were daily examined for the presence of cytopathic

effect (CPE) typical of AMPV. After 6 days post infection (d.p.i.) the cell sheets were freeze-thawed and the material was used to infected new cell sheets. The new infected cell sheets were viewed daily for the presence of CPE up to 49 d.p.i. and a third passage was performed. Eventually, to check the presence of rescued viruses, RNA was extracted (mc 3.1) from the Vero cell monolayers of the third passages, reverse transcribed (mc 3.2) and amplified by PCR (mc 3.3).

Name	**Sequence (5'…3')**	**Function**
Cassette +	TCGACGGGACAAGTCGACAGTAATTAAAAAAG	Cloning cassette
Cassette neg	TCGACTTTTTTAATTACTGTCGACTTGTCCCG	Cloning cassette
M-F Xho +	GTCCACTATTCTGTAGTTTAATAAAAACTCGAGGGGGCA AGTAAAATGTACCTCAAACTGCTAC	Introduction XhoI site at M-F
M-F Xho neg	GTAGCAGTTTGAGGTACATTTTACTTGCCCCCTCGAGTTT TTATTAAACTACAGAAGAATAGTGGAC	Introduction XhoI site at M-F
B 2.87 +	CCAGAGAACTAGGTATGTCC	Cassette at M-F amplification
B 3.23 neg	CCTATGGGAAAGGATTCGATTC	Cassette at M-F amplification
G13+B	CAATCCTAGTCAATCGGGAACC	Cassette at G-L amplification
B 7.46 neg	GGTATGGTCGTCCTATAATGCAAGATCC	Cassette at G-L amplification
RT 26.24 neg	CCAAGATACATTTCCAG	M reverse transcription
M IBV Xho +	TCCAGCAAATCTCGAGGATGTCCAACGAGACAAATTGTA	M amplification Xho I site
M IBV Xho neg	TCTCTACACACTCGAGTTTATGTGTAAAGGCTACTTCCAC TTG	M amplification Xho I site
IB 25.27 +	GACGTAATATCTATCGTATGGTGCAG	M screening
N all neg	ACTAATGAGAATCACAATAATAAAAAGCACAG	N reverse transcription
N Xho start +	AAGGGACAACTCGAGCATGGCAAGCGGTAAGGC	N amplification XhoI site
N Xho end neg	CTTTTTTTCATAACTACTCGAGTCAAAGTTCATTCTCTCC	N amplification XhoI site
N end +	GATGATGAACCAAGACCAAAG	N Screening
GAB 1 +	GGCTTGACGCTCACTAGCACTATTG	S1 at G-L amplification
B 7840 neg	CATCTCTGCAGCATTGGACATATCG	S1 at G-L amplification
S1 end +	GCTGTTAGTTATAATTATCTAG	S1 Screening
S1 675 +	GGATCACCTAGAGGCTTGTTAGC	S1 Sequencing
S1 765 neg	CACGATAGACAATAAACTTCTGCTTAAC	S1 Sequencing
MAB 3+	GAGAGCTTAGGGAAAATATGCAAAACATGG	Insert genes sequencing
FAB 4 neg	CTCAACTGATGTAGCCCATGTTGC	Insert genes sequencing
GAB 4 +	GCTGATTGAGTGGTGCTGTACTAG	Insert genes sequencing
B 7.40 neg	GGAGTCAGGCAGATACACATTCACCG	Insert genes sequencing
Dta-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTA	mRNA reverse transcription
Dtc-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTC	mRNA reverse transcription
Dtg-Adaptneg	GCATCTCGAGGCTTGTGGCTTTTTTTTTTTTTTTTTTTTG	mRNA reverse transcription
Adaptneg	GCATCTCGAGGCTTGTGGCT	mRNA amplification

Table 7.1 List of the primers used in the present study.

7.3 RESULTS

7.3.1 Genes addition

The cloning cassette was added at the M-F intergenic region of cvB plasmid and the N gene amplicons was ligated into it. Using PCR and restriction endonuclease (RE) digestion, one FL plasmid containing the N gene was found. And was named $cB_{N\ M\text{-}F}$.

The cloning cassette was also ligated at the M-F junction of plasmid $cB_{S1+IL\text{-}18\ G\text{-}L}$ and the N and M genes were ligated into it in two different reactions. Four FL plasmids containing the N gene in the M-F intergenic region were found positive both to PCR and RE screening and four plasmids were positive for the M gene insertion.

The S1 gene was added by site directed mutagenesis (SDM) at the G-L intergenic region of plasmid $cB_{N\ M\text{-}F}$, as demonstrated by the gene being detected in three clones by PCR and RE screening.

The cloning cassette was added by SDM at the G-L junction of $cB_{N\ M\text{-}F}$ and M gene amplicons ligated into it. Eight clones containing the M gene at G-L junction and the one clone containing the N gene at M-F were detected by PCR and RE screening.

Sequencing analyses confirmed that no mutations were present in the exogenous genes of any clones.

7.3.2 Recovery of recombinant viruses

Several rescue attempts were performed for each constructs, but none generated virus.

7.3.2.1 $cB_{N\ M\text{-}F}$ rescue attempts

Vero cells monolayers transfected with construct $cB_{N\ M\text{-}F}$ were viewed daily for the presence of cytopathic effect (CPE). After 6 days post infection (d.p.i.) on the second passage no CPE was detected. After 25 d.p.i. cellular changes were observed; but not clear CPE. After 31 d.p.i. areas similar to CPE was observed. A further passage was performed, but no CPE was detected. Potential avian metapneumovirus (AMPV) RNA was extracted from the cell monolayer but viral mRNA wasn't detected.

7.3.2.2 $cB_{N\ M\text{-}F\ S1+IL\text{-}18\ G\text{-}L}$ and $cB_{M\ M\text{-}F\ S1+IL\text{-}18\ G\text{-}L}$ rescue attempts

Constructs $cB_{N\ M\text{-}F\ S1+IL\text{-}18\ G\text{-}L}$ and $cB_{M\ M\text{-}F\ S1+IL\text{-}18\ G\text{-}L}$ were transfected into Vero cells. After 28 d.p.i. some areas of the second passage showed cellular changes. After 49 d.p.i. clear CPE was not detected and a third passage was performed. After 24 d.p.i on the third passage the Vero cell monolayers didn't show any signs of CPE. RT-PCR didn't detect any AMPV mRNA.

7.3.2.3 $cB_{N\ M\text{-}F\ S1\ G\text{-}L}$ rescue attempts

The construct was transfected into Vero cell monolayers and viewed daily for CPE. After 30 d.p.i, the second passage contained a few areas of cellular change. A third

passage was performed, but after 24 d.p.i no sign of CPE was detected. RT-PCR against AMPV viral mRNA was negative.

7.3.2.4 $cB_{N\ M\text{-}F\ N\ G\text{-}L}$ rescue attempts

The construct was transfected on Vero cell monolayers and viewed daily for CPE. At 23 d.p.i., the second passage contained some areas cellular changes. After 48 d.p.i. a third passage was performed, but after 24 d.p.i. no sign of CPE was seen. RT-PCR direct against AMPV didn't detect viral mRNA.

Clone name	Insert gene	Position	No. Plasmids	Rescued/attempts
$cB_{N\ M\text{-}F}$	N	M-F	1	0/1
$cB_{N\ M\text{-}F+S1\ G\text{-}L}$	N and S1	M-F and G-L	3	0/3
$cB_{N\ M\text{-}F+S1+IL\text{-}18\ G\text{-}L}$	N, S1 and IL-18	M-F and G-L	4	0/
$cB_{M\ M\text{-}FS1+IL\text{-}18\ G\text{-}L}$	M, S1 and IL-18	M-F and G-L	4	0/4
$cB_{N\ M\text{-}F+M\ G\text{-}L}$	N and M	M-F and G-L	8	0/8

Table 7.2 Summary of the constructed recombinant cloned FL cDNAs

7.4 DISCUSSION

In the present study several rational attempts to increase the ability of subtype B recombinants to induce protective immunity were performed as based on the data obtained from previous chapters and from a study of subtype A recombinants (Falchieri et al., 2013). Several constructs were obtained, containing up to three exogenous genes in two different intergenic regions, but none of them was rescued, despite several attempts and longer passages on Vero cells.

The plasmids transfected into Vero cells were all based on the hybrid genome copy generated in a previous study, but they differ in the number of exogenous genes inserted. The common feature between all the plasmids was the presence of an exogenous gene in the M-F intergenic region, strongly suggesting that the insertion of a foreign gene at that particular intergenic region compromised the virus viability of subtype B recombinants. These data contrast with what has been observed for subtype A, where the M-F junction represented the best compromised between exogenous gene expression and virus viability (Falchieri et al., 2013). A different behaviour between the two subtypes is the most likely explanation for the conflicting results obtained in the two studies, but also other conclusions might be drawn. In their study, Falchieri et al. (2013) cloned QX N or QX S1 genes at the M-F junction, while in the present study Massachusetts (Mass) N or Mass M genes have been cloned. It is unlikely that the different strains or the different genes used in our study caused the lack of virus viability, but at the moment this hypothesis cannot be fully put aside. In the previous chapter we have observed that the rescue of a plasmid containing the IL-18 gene and an IBV gene in the same intergenic region, can only be achieved when the IBV gene was the S1 and not the N, suggesting that a particular sequence or a particular combination of sequences can prevent the recovery of the virus. These could be easily verified inserting QX S1 or QX N gene in the M-F intergenic region of the hybrid subtype B plasmid and subsequently attempting the virus rescue. Nevertheless a more rational approach could be adopted to verify these two hypothesises and to obtain at the same time useful information for further studies. The construction of seven subtype B hybrid plasmids containing a reporter gene in a different intergenic region would determine the viability of each virus and of the exogenous gene expression with respect to every intergenic region.

As previously mentioned, the failure to recover virus containing the M gene makes it impossible to currently evaluate the ability of the M protein to stimulate a protective immune response. To most conveniently address this, it should be possible to insert the M gene at the G-L junction of the hybrid AMPV genome copy and testing this in homologous IBV challenge studies.

The flexibility of making different constructs confirmed the suitability of AMPV subtype B as a vector for the development of recombinant vaccines. Up to 4000 exogenous nucleotides have been inserted in two different intergenic regions of subtype B genome. Nevertheless, the insertion of an exogenous gene at the M-F junction seems to have compromised the virus viability causing failure in the recovery of the recombinants. Future studies should focus on subtype B recombinant viability, as such studies would allow us to determine the intergenic regions showing the best balance between virus viability and exogenous genes expression. That knowledge could be crucial in the development of effective subtype B recombinant vaccines.

8. CONCLUSION

Reverse genetics (RG) was first applied to avian metapneumovirus subtype A and C (Govindarajan et a., 2006; Naylor et al., 2004), and different studies investigated the effects of single and multiple genomic mutations (Brown et al., 2011; Naylor et al., 2006; Naylor et al., 2013) gene deletions (Ling et al., 2008; Yu et al., 2011) and insertions (Govindarajan et al., 2006; Lupini et al., 2008) on virus phenotype. To extend this to subtype B, several attempts had been previously made to establish a subtype RG system but these failed. In the current study, a RG system for subtype B was developed for the first time (chapter 4), then used to investigate subtype B as a vector for the expression of infectious bronchitis virus (IBV) genes (chapter 5). Attempts to confer the protection induced by the recombinants against IBV was described in chapters 6 and 7.

In chapter 4 a comparison was made of the AMPV subtypes A and B amino acids sequences. The analysis focused initially on the genes forming the Ribonuclear Complex (RNP), directly involved in the RG system (Naylor et al., 2004) and showed there to be high identities and similarities between the two subtypes. When compared to subtype C, less identity and similarity were observed. The analysis was extended to the leader and trailer nucleotide sequences, and to the gene transcription start and stop sequences. The first 12 bases of the leader of all the three subtypes and of subtype A trailer were identical, while 2 mismatches were observed in subtype B and C. A common antigenome sequence was also found in the trailer of all the subtypes, between nucleotide 13-21. More studies need to be done to understand the role of these sequence. A common transcription start sequence was observed in the three subtypes, with the exception of the L gene, and of the G and SH genes in subtype B. The analysis of the transcription stop detected some slight differences between subtypes: nevertheless, the differences observed were no greater than those identified within subtypes. The data indicated that subtypes A

and B might be recovered using support genes of both the subtypes. Plasmids containing the B type support genes, as well as plasmids containing the full genome copy of a subtype B virus were obtained. Virus was rescued from subtypes A or B full length genome copies using either A or B support plasmids. These data confirmed that the polymerase of either subtype can recognise leader, trailer and gene start and stop sequences. For the first time a subtype B virus was recovered. The differences detected in subtype C when compared to subtypes A and B are greater to those found between subtypes A and B, hence it is not clear if a common RG system might be possible.

Chapter 5 described the construction of two subtype B recombinant viruses, carrying the S1 and the N genes of IBV. The S1 and N proteins had previously proved able to induce protective immunity (Cavanagh. 2007; Seo et al., 1999; Yu et al., 2001). The genes were inserted into the intergenic region between the G and L genes of AMPV and recombinant viruses were successfully rescued. AMPV subtype B proved able to replicate and transcribe the exogenous genes *in vitro*. *In vivo* the recombinants gave poor protection, assessed by observing tracheal cilial motility after virulent challenge with IBV. Recombinant replication in the respiratory tract was poor, and a serological responses against AMPV were largely absent. The poor replication in the trachea was associated with negligible protection. To increase replication and possibly protection, the genes involved in attachment and release of the virus from the host cell were modified and a chimera full length AMPV subtype B sequence was obtained (see chapter 6). S1 and N sequences were cloned into the chimera subtype B and chimera recombinant viruses were rescued. The replication in the trachea was very high and the protection induced by subtypes B chimera recombinant was generally improved in comparison to subtypes A (Falchieri et al., 2013) and previous subtype B recombinants (chapter 5). A better protection was observed in birds vaccinated with recombinant AMPV expressing

the N gene, suggesting that this protein is the major immunogenic protein of IBV. Nevertheless the pharmacopeia efficacy requirement was not met. It remains unclear whether the failure to confer full protection might be attributed to the low exogenous gene expression or perhaps the lack of expression of as yet unrecognised immunogenic IBV proteins.

In chapter 6 interleukin 18 (IL-18) was added in the chimera plasmids downstream the IBV gene, as IL-18 stimulates the release of interferon ɣ (IFN-ɣ) (Schneider et al., 2000) and has been shown to enhance vaccine efficacy (Göbel et al., 2003; Winfried et al., 2004). Only virus containing both the S1 and the IL-18 genes was rescued. Chickens inoculated with this recombinant were challenged *in vivo*, and found to be better protected than recombinants expressing only the S1. Surprisingly it was not possible to rescue virus expressing N and Il-18 proteins. It's likely that the presence of these two foreign genes in the same intergenic region removed virus viability.

In chapter 7 further attempts to increase the protection conferred by the recombinants were described. The N gene was inserted at the M-F junction to increase the protein expression without affecting virus viability, as suggest by a study carried on subtype A recombinants (Falchieri et al., 2013). That same study indicated that the coexpression of more than one IBV genes conferred better protection (Falchieri et al., 2013). Several plasmids containing two IBV genes were obtained. None of plasmids generated successfully produced virus. The presence of an exogenous gene at the M-F junction might have compromised the virus viability. Further studies need to be done to fully understand the subtype B viability in respect to the site of insertion of the exogenous genes.

In conclusion, the first AMPV subtype B RG has been developed. It has been shown that subtypes B and A support protein genes can rescue both subtype B or A full

length genome copies. AMPV subtype B proved tolerant in accepting extra genes at intergenic positions and several recombinants viruses were successfully rescued. Inserted nucleotide sequences were conserved during passage *in vitro*, and transcription and expression of foreign genes were demonstrated. Nevertheless the viral viability appeared to be affected depending on the site of insertions. Furthermore, the size limit of inserted exogenous nucleotides is still not determined. The recombinant viruses have been tested as candidate vaccines in chickens against IBV and despite some promising results, more studies needs to be done in order to develop efficacious AMPV-B/IBV recombinant vaccines.

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