Avian metapneumovirus studies using Reverse Genetics

Thesis submitted in accordance with the requirements of the

University of Liverpool

for the degree of

Doctor in Philosophy

By

MARCO FALCHIERI

SEPTEMBER 2012

Brisa strazér i marón!

TABLE OF CONTENT

ABSTRACT

Avian metapneumovirus (AMPV) is an enveloped negative sense single stranded RNA virus which is a major endemic respiratory pathogen of global domestic poultry. Since reverse genetic (RG) techniques have been applied to this pathogen several reports have investigated the effects of single and multiple genomic mutations and gene deletions or insertions on viral biology. The aim of this study was to gain a better understanding of the viral capacity to accept and in some cases express homologous and heterologous extra sequences.

Initially an AMPV subtype A was modified to introduce a homologous 200 bp sequence within the G gene and this recombinant was suggested to be used as a positive control for validating all stages of a previously established RT-nested PCR.

Different Green Fluorescent Protein (GFP) AMPV recombinants were then prepared each one containing the reporter gene in a different intergenic position and then were assessed for expression, stability and viability. In particular, quantification of the expression was calculated using a quantitative ELISA. All recombinants showed high stability, while good viability was observed in all the positions, except when GFP was inserted between nucleocapsid (N) and phosphoprotein (P). The highest expression was detected in the virus with the insertion between N and P as expected, according to the transcriptional model for non-segmented negative stranded viruses. However GFP was produced at high levels even when inserted at the trailer end intergenic positions. Poor expression was seen for all the other positions.

The vectoring abilities of subtype A strains were further investigated to accept and express foreign genes, specifically GFP gene and both spike (S1) and nucleocapsid (N) genes of infectious bronchitis virus (IBV). After viruses had been recovered by RG, all recombinants were proven to express the inserted genes efficiently and were all found to be highly stable during passage *in vitro*. Subsequently IBV recombinants were tested as candidate vaccines by eye-drop inoculation of one-day-old chickens. When chicks were challenged with IBV, partial protection results were observed, as assessed by greater motility of tracheal cilia from animals receiving the recombinants.

Finally the development of a new RG system was attempted in order to extend this type of studies to the B subtype. This subtype is distributed worldwide and growing field evidence suggests it to be more able to infect commercial chickens compared to subtype A. For this reason it would be convenient to have an RG available also for B viruses. The construction of a DNA copy of the viral genome was attempted using site-directed mutagenesis and ligation techniques, resulting in more than 85% of the genome cloned. Unfortunately full genome cloning proved to be not possible, as severe problems were encountered, including construct instability and cloning bacteria intolerance to viral sequences.

INTRODUCTION

In the late 1970 in South Africa a severe pathological condition of turkeys, characterized by an acute rhinotracheitis, hit the local poultry industry heavily (24). Although many previously known respiratory pathogens could have been responsible for such a condition, no immediate diagnosis was made. Subsequent analysis pointed to an unknown viral agent, promptly named *Turkey rhinotracheitis virus* (TRTv), as cause of that epizootic (24). The virus was later charachterized as a negative sense, non-segmented, single stranded RNA virus closely related to mammalian respiratory syncytial viruses (178). For this reason it was classified within the *Paramyxoviridae* family, subfamily *Pneumovirinae*, genus *Pneumovirus (178)*. More recently, following the detection of a similar virus infecting humans, TRTv was then placed in a new genus, *Avian metapneumovirus* (AMPV) (224). After its first detection in Africa (24) AMPV spread rapidly to Europe (7) and then to most parts of the world (50), becoming immediately a major problem for turkey production, but also for chickens, which soon proved to be susceptible to it. Losses were mainly due to a decreased bird growth rate and sometimes to high mortalities caused by secondary bacterial infections (205). Furthermore field and experimental evidence showed the viral capacity to affect egg production in laying birds, both in terms of number of laid eggs and egg quality (69, 190). Up to now AMPV control has been possible only by using live attenuated and killed vaccines (89). However, this approach has been shown to be limited, especially regarding eradication; also live vaccines have sometimes been shown to revert to virulence in turkeys (30) and as different viral subtypes are present, protection is often limited to certain strains (51). On the other hand killed vaccines appeared to be ineffective in preventing the respiratory infection, and are mainly administered to protect against egg drops (51).

Due to the unsatisfying results obtained using the above mentioned strategies, researchers have started to apply new generation techniques, not

just to develop better vaccines but even to reach a better understanding of viral biology (91, 123, 158, 179). In 2004 the first AMPV reverse genetic system (RG) was developed (158), enabling the recovery of the RNA virus from a DNA copy (cDNA) of its genome. As DNA molecules are more stable and easily modifiable than RNA ones, RG has given the opportunity to scientists to introduce not only single or multiple mutations, but also deletions and insertions into the AMPV genome and, followed by viral rescue, to readily observe the effects of those modifications on virus biology.

In 2004 an RG system was specifically developed for subtype A of AMPV (158). This thesis has investigated the ability of this subtype to accept, tolerate and express inserted foreign sequences. Moreover an attempt to adapt this technique to subtype B was made.

In Chapter 2, a homologous M2 gene sequence of approximately 200 bp was introduced into the G gene using restriction endonuclease cut properties and ligation steps. The insertion was made in a such way as to increase the size of a common AMPV diagnostic RT-PCR amplicon of the G gene. Rescued virus proved to be unaffected, at least *in vitro*, by the insertion, and it was proposed as positive control for diagnostic PCRs.

Chapter 3 investigates the positional effects of inserts on AMPV. Current transcriptional models for non-segmented negative stranded RNA viruses suggest 3’ upstream genes to be transcribed more efficiently than downstream ones. As polymerase entries the genome only in that position, messenger RNAs are produced in a polar and sequential way, which gradually decrease due to polymerase dissociation. This results in a major transcription and then protein synthesis of genes close to the 3’ end compared to the 5’ end ones. Seven different recombinants carrying GFP were constructed, each one expressing the insert in a different intergenic position. Recombinant viruses were passaged, sequenced and finally titrated according to two different

methods. GFP expression was verified both by fluorescence observation and by specific ELISA. The latter enabled not only the detection of GFP but also quantification of the amount of protein produced.

Chapter 4 describes the modification of the AMPV genome to be able to accept and express foreign genes. Extra gene transcriptional starts and stops were included into different intergenic regions so that genes could be inserted. To assess the system, green fluorescent protein was initially added in different positions. Once expression was verified, observing the fluorescence under UV microscope, AMPV recombinants carrying infectious bronchitis virus (IBV) S1 and nucleocapside genes were constructed. After *in vitro* studies assessing foreign gene expression, stability, viability and cytopathogenicity, recombinant viruses were used as vaccine candidates against IBV in chicken experimental infections.

Finally, Chapter 5 describes the attempt to construct a subtype B reverse genetics system. While for A and C subtypes such a technique has been successfully applied, for subtype B, despite several efforts having been made, RG has not yet been successful. As a first step a cDNA of a full length B subtype genome had to be constructed by cloning viral PCR amplicons in a plasmid, followed by the construction of plasmids encoding for essential AMPV support proteins. This procedure has proved to be very critical in view of plasmids instability and intolerance of the cloning bacteria to certain viral sequences.

The thesis is opened by an initial chapter (Chapter 1) reviewing all the general aspects regarding *Avian metapneumovirus*, as described by the innumerable studies which have been carried out since AMPV presence was revealed about 30 years ago. As science is formally communicated through scientific articles, which have usually a fixed and formal structural organization, including introduction, material and methods, results and discussion paragraphs, we have decided to write every chapter as an independent paper,

each one with its own sections. A further chapter, stating final discussions, closes the thesis.

CHAPTER 1

AVIAN METAPNEUMOVIRUS LITERATURE REVIEW

TABLE OF CONTENT

Page

1.1 AETIOLOGY

1.1.1 Taxonomy

Avian metapneumovirus (AMPV) is one of the two viral species, with human metapneumovirus (HMPV), belonging to the *Metapneumovirus* genus; Metapneumoviruses are part of the subfamily *Pneumovirinae* within the *Paramyxoviridae* family, including single stranded, negative sense RNA, and enveloped viruses (178, 224, 226).

1.1.2 Morphology

Viral particles are characterized by high pleomorphism (Figure 1), both in size and length; virions can be spherical or have a filamentous form, usually of 40 – 500 nm, but sometimes even over 1000 nm. Their nucleocapsids have a helical shape and on the envelope surface projections of about 13 – 14 nm are clearly distinguishable (11, 25, 46, 51, 84, 86, 145, 240).

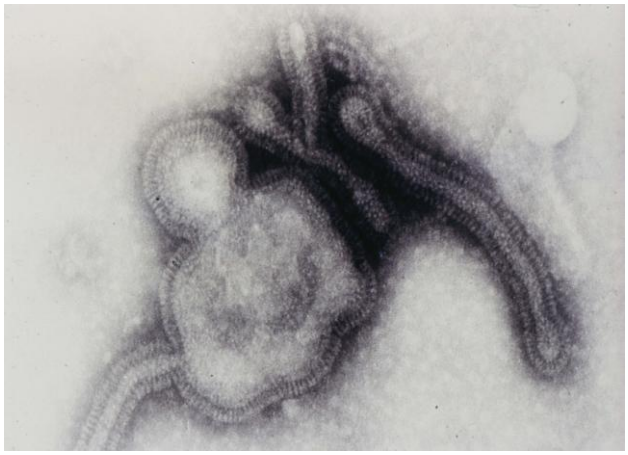

Figure 1: AMPV observed under electronic microscope

(provided by Prof. R.C .Jones)

1.1.3 Genome

The viral genome consists of a single negative stranded RNA of about 13000 - 14000 nucleotides (181). It encodes for 8 genes (Figure 2) which lead to the synthesis of at least 9 proteins: starting from the 3' end, we can recognize in order N, P, M, F, M2 (including two overlapping open reading frames), SH, G and L (70, 132). Every gene is flanked by a transcriptional start sequence and a transcriptional stop, and between each transcriptional units there are intergenic untranslated regions. At the leader (3') and trailer (5') ends, other untranslated sequences of about 40 nucleotides are known, containing promoters and regulatory sequences for transcription, replication and packaging (91, 132, 233).

Figure 2: AMPV genome

1.1.4 Proteins

Each gene encodes for the homonym protein. The Nucleocapsid protein (N) forms the integral part of the nucleocapsid: tightly joined to the genome, it provides the typical helical structure. N is an essential part, with the Phosphoprotein (P) and the Polymerase (L), of the ribonuclear complex. L and P are therefore critical for replication and transcription, and they function respectively as a catalytic and non-catalytic factor in these processes. The Matrix protein (M) is situated in the inner envelope surface, anchoring the nucleocapsid to the lipidic membrane. AMPV has got an accessory Matrix gene (M2), encoding for two different proteins: M2-1, which seems to behave as a transcription elongation factor and M2-2, which has shown transcription

inhibition properties. The small hydrophobic protein (SH) is an integral membrane polypeptide; however its function is poorly understood. The Fusion (F) and the Attachment (G) proteins are located on the external part of the envelope and are the major antigenic determinants (21, 48, 70).

1.1.5 Virus Attachment and Entry

Viral particles interact with the host cells using the surface proteins (131). The G protein enables virus attachment on the cell receptors, while F promotes the fusion of the envelope with the cell membrane; the nucleocapsid is then released in the cytoplasm, where AMPV replication occurs (70, 77).

1.1.6 Replication and Transcription models

AMPV follows the generally accepted replication and transcription models for other Mononegavirales. While plus-stranded RNA viruses possess a genome able to function immediately as a messanger RNA, and for this reason able to synthetize immediately all viral proteins, negative single stranded RNA viruses require the ribonuclear complex both for transcription and replication. This means their genome needs to be encapsidated with the nucleoprotein (N) and associated with the RNA dependent polymerase (L) and its cofactor (P) to initiate the infectious cycle. The polymerase can enter the genome only at the 3' leader end, and as it moves along the RNA, it encounters a transcriptional start signal; a messenger RNA (mRNA) starts then to be synthetized and the process stops when a transcriptional stop signal is met. The polymerase molecules at this stage have got two possibilities: half of them will dissociate from the genome and will rebind again from the 3'end, and the other half will move along and at the next transcriptional start will begin to transcribe a new

gene. This mechanism regulates all the transcription process until the end of the viral genome, meaning a good number of polymerase molecules will dissociate at every gene junction. This results in a sequential and gradually decreasing mRNA production: the genes located proximally will be transcribed in higher quantity compared to the ones placed distally. A minor or major amount of mRNA will result in a minor or major protein production (68, 229, 233).

Replication starts with the synthesis of a complementary positive copy of the genome, called antigenome. This is used as a template for genome production. During replication the polymerase ignores the transcription start and end signals, reading fully through all the viral RNA. The mechanisms that permit the polymerase to behave differently during transcription and replication are not clear, but some studies on human respiratory syncytial virus indicate that the concentration of N may be critical in regulating this process (76, 91, 233).

1.1.7 Packaging and Assembly

Nucleocapsid formation starts with the association of viral RNA with the N protein, immediately followed by P and L (126). This leads to the formation of the ribonuclear complex, that is the functional unit of the virus both for replication and transcription (229). The M protein concentration seems critical in final assembly, as this protein is able to interact firstly with the ribonuclear complex (including N, P, L and the viral genome), located in the cytoplasm, and then with the surface proteins (G, F and SH), which after synthesis, have been inserted into the cell membrane; these last interactions, in particular, results in virions budding from the cell surface (70, 171, 210).

1.1.8 Strain Classification

Four subtypes of AMPV have been identified: A, B, C and D. Early studies, which showed differences among AMPV strains using monoclonal antibodies (45, 57) and sequencing and amino acidic analysis (122, 157), suggested the existence of two subtypes, A and B. In 1997, following the first North American AMPV outbreak (193), subtype C was identified. Both serological (55) and genomic techniques (194, 195) have highlighted the differences with the previous subtypes. Finally retrospective analysis of French strains, isolated in 1985, resulted in the identification of subtype D (12, 213).

1.1.9 Physical and Chemical Susceptibility

AMPV is stable at pH range from 3.0 to 9.0, sensitive to lipid solvents and inactivated at 56˚C for 30 minutes (46, 89). Subtype C is reported to be resistant to cold temperatures (at -70 and - 20 ˚C for more than 26 weeks), but a viral survival is dramatically decreased at higher temperatures (only 6 hours at 50˚C). Many disinfectants, e.g. ethanol, ammonia, iodophor, phenol derivates and hypochlorite, are effective against AMPV. Moreover the virus was resistant to drying for 7 days (217) but could survive for several days in turkey litter at different temperatures (227). Finally more recent studies have proposed microwaving, autoclave treatment (71) and high pressure processing as convenient and easy methods for viral inactivation (134).

1.2 EPIDEMIOLOGY

1.2.1 Distribution

AMPV has been detected worldwide, with the only exception of Oceania (14). The disease appeared for the first time in South Africa in the late ’70 (25), and then spread to Europe (6, 7, 18, 19, 73, 75, 99, 150, 177), Asia (82, 135, 168, 221, 231), South and Central America (8, 66, 115, 216) and other African countries (26, 105, 169). In North America it has been reported only since 1996 (198).

Subtypes A and B are responsible of the disease in all affected continents, with the only exception of North America, where infections are caused just by AMPV subtype C. Subtypes C related strains have been more recently detected in France (212) and Korea (130). Subtype D was isolated only once in France in 1985 (12).

1.2.2 Hosts

Turkeys and chickens of all ages are the natural hosts (89). In particular the first species seems to be the most susceptible. Guinea fowls and pheasants are also sensitive to AMPV infections (33, 87, 88, 175), while Pigeons, geese and ducks were believed to be resistant (87); however recent studies have suggested a sensitivity of waterfowls to subtype C (203, 215). Antibodies against AMPV have been detected also in farmed Ostrich in Africa (26). Wild birds sensitivity to AMPV has been proved only for subtype C (15, 16, 203, 220), while for subtype A and B this issue remains poorly understood (67, 101). Avian Metapneumovirus is not considered a zoonotic pathogen but recent serological

surveys suggest that occupational exposure to turkeys might be a risk factor for human infection (124).

1.2.3 Transmission

Direct contact transmission seems to be the only demonstrated way of infection, both directly with infected animals or their respiratory discharges (2, 5, 61, 145). Other transmission ways have been supposed (201), e.g. vertical (121, 125) or through contaminated water, equipment, feed truck or personnel, but no real evidence was demonstrated until now (115). In the USA migratory species have been recognized as a possible viral source, although transmission between wild and domestic birds remains to be proved (89).

1.3 PATHOGENESIS

The upper respiratory tract is considered to be not just the first replicative site of the virus but also the main target tissue for viral replication. AMPV seems to have a particular tropism for the ciliated cells of the nasal cavities, conchas, infraorbital sinus and trachea. In these organs viral particles can be detected until 7-9 days post infection using immunofluorescence technique (121) and isolated until 14 days post infection (53) in turkeys. Similar findings have been demonstrated also in chickens (32). However, occasionally AMPV can reach the lungs and the air sacs (5, 32, 58, 139). Bacterial co-infections seem to facilitate viral penetration along the lower respiratory tract: *Escherichia coli* (1, 219, 223), *Bordetella avium* (61, 113), *Mycoplasma gallisepticum* (156) and imitans (80), *Riemerella anatipestifer* (187), *Chlamydophila psittaci* (225), *Ornithobacterium rhinotracheale* (142) have all proved to exacerbate the disease and to enhance virus distribution in infected birds. On the other hand, concurrent infections with infectious bronchitis virus appeared to inhibit replication of AMPV (118). It is still not clearly understood how the virus can infect other organs outside the respiratory system, but it is common to detect AMPV mainly in the reproductive tract (49, 104, 121, 206, 228) and on some occasions in the Harderian gland, kidneys (125), spleen, cecal tonsil and bursa of Fabricious (9). A short transient viraemia could explain this behavior, although AMPV has been rarely found in the circulation (202). Some authors have suggested, supported by in vitro studies with subtype C, that macrophage cells are particularly susceptible to AMPV and to possibly be responsible of viral dissemination (200). No clear differences have been found in the pathogenesis among different subtypes: different results seems more

related to the virulence of the single strain than to the subtype belonging (9, 202, 222).

1.4 DISEASE IN BIRDS

The clinical picture of AMPV infection is typically characterized by respiratory symptoms: coughing, sneezing, nasal discharge, swollen infraorbital sinus, but even conjunctivitis and submandibular oedema can be present (25, 117, 145). The disease is also called turkey rhinotracheitis (TRT) as this species appears to be more severely affected compared to chickens, where often the infection is asymptomatic (50). Morbidity is usually very high (almost 100%), while mortality can be very variable (172). The severity of the disease is highly dependent on management factors, including bird density, ventilation, temperature, hygienic conditions, and on secondary bacterial infections (89, 96, 205). Co-infections of AMPV and *E.coli* have been associated in chickens with swollen head syndrome (SHS). This is characterized not just by respiratory signs but also of a general head swelling (Figure 3), which leads to neurological signs, such as disorientation, torticollis and opistothonus (96, 120).

AMPV infections cause drop in egg production in laying birds. Drops in egg production are usually of the order of 10-20% (190), which can reach even 70% (239) in turkeys. Egg quality is affected, showing poor and thin shells (69). Drops in egg production have been reported in field in laying hens too, although in experimental conditions have never been seen after experimental respiratory challenge. In contrast to the situation in turkeys, only intravenous injection of virus is able to decrease the laying performance in chickens (52, 104, 206). This difference between the two species has never been explained.

Figure 3: SHS in chicken (provided by Prof. E. Catelli)

1.5 POST-MORTEM FINDINGS

1.5.1 Gross Lesions

While in turkeys gross lesions, due to uncomplicated infections, are a considerable finding, in chickens these are quite uncommon (89). In turkeys, lesions include the presence of watery to mucoid exudate in the upper respiratory tract, swelling of the infraorbital sinus caused by accumulation of mucus, conjunctivitis and submandibular oedema (205). In breeders, prolapsed oviducts, folded shell membrane in the reproductive tract and egg peritonitis may be seen (121). Bacterial secondary infection can aggravate these findings, resulting in airsacculatis, pericarditis and perihepatitis (205). In chickens, during SHS presence, head and neck may increase in size and be swollen, due to an accumulation of a yellow gelatinous or even purulent oedema in the subcutaneous tissue (176).

1.5.2 Microscopic Lesions

No major differences have been found between turkeys and chickens. As said previously AMPV has a particular tropism for epithelial cells. The main histological lesions are located on the respiratory epithelium, characterized by deciliation, deepithelization, thickening of the mucosa, hyperaemia, mononuclear infiltration and glandular proliferation in the turbinates, infraorbital sinuses and trachea (Figure 4). Lesions are usually transient and detectable in the first 10 days after infection. After 3 weeks, birds are totally recovered (9, 32, 114, 222). Epithelial damages in the oviduct have been also observed (52).

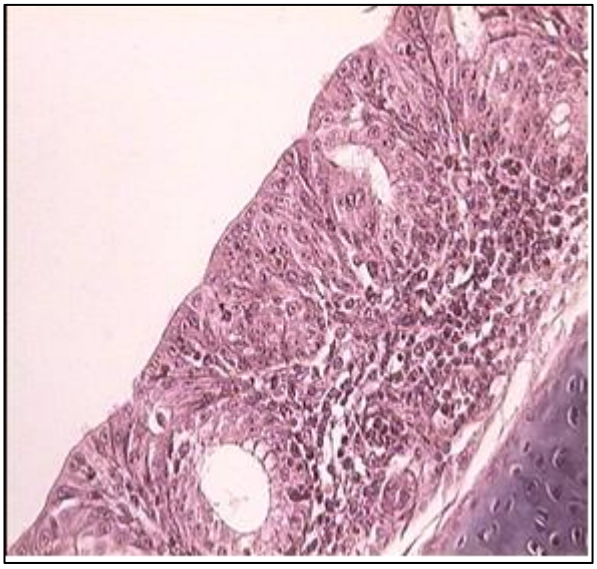

Figure 4: microscopic lesions in trachea after AMPV infection (provided by Prof. E. Catelli)

1.6 IMMUNITY

The immune reactions of turkeys towards AMPV infections have not been fully clarified. Some studies speculated cellular mediated immunity to be critical for protection, while humoral immunity appears to be not critical, and circulating antibody titers do not seem to be an indicator of protection (182). In experimental infections, turkeys with no detectable antibodies were protected against challenge with a virulent strain (62) and vaccinated bursectomised poults were resistant to challenge (119). Furthermore suppression of T-lymphocytes with cyclosporine A caused delayed recovery from clinical signs and more lasting microscopic lesions (186). Local immunity could be related in resistance to infection, however, as suggested for other respiratory pathogens (79), its short duration might explain recurrent infections during birds productive life in farms (182). Maternal antibodies are passed from hens to their progeny via the egg yolk, but their role does not seem to be significant, as they do not prevent infections (162) or do not interfere with early vaccination, allowing young chicks to be immunized in early stages (62) or directly *in ovo* (237).

1.7 DIAGNOSIS

Both clinical and post-mortem findings are not specific for AMPV as they can be similar to those caused by other viral respiratory pathogens such as Newcastle disease, infectious bronchitis, low pathogenic avian influenza, mycoplasmas or respiratory bacterial infections. Viral identification is therefore

critical for a definitive diagnosis. This target can be reached directly by isolating or detecting the virus, or indirectly, by demonstrating specific serological responses in the host (90).

1.7.1 Direct Diagnosis

AMPV has a very short persistence in the host before clearance, both in turkeys (53) and chickens (32). For this reason virus isolation and detection are not always easy. Samples must be taken in the very early stages (at 3-5 days after infection) from birds not yet showing clinical signs (51).

1.7.1.1 Isolation

Primary isolation of AMPV is possible using tracheal organ culture (TOC) (145) or embryonated eggs inoculated via the yolk sac (25, 170). Both methods seem to have good sensitivity (51), but isolation of subtype C in TOC is not suitable as this strain does not cause ciliostasis (55). Once isolated, AMPV can be easily adapted to grow in different cell lines such as Vero (25) or in chick embryo fibroblasts (94) and chick embryo liver cell monolayers (235). The cytopathic effect (CPE) is not specific and often characterized by the presence of small cyncytia (25, 112). Other cell lines have been suggested both for primary isolation and viral passages, such as QT-35 (93), primary turkey turbinate (127), turkey and chicken kidney cells (127), BGM, DF-1 (211), CER and BHK-21 (64), but are not commonly in use.

1.7.1.2 Viral Detection

Different immunochemical methods have been used to detect viral antigens in fixed and unfixed tissues and smears (89). Immunoperoxidase (32, 111, 139), immunofluorescence (116, 121) and immunogold staining (165) have been widely used during scientific studies. Specific monoclonal antibodies and immunodiffusion tests have been also used to differentiate between viral subtypes (45, 57, 90). All these methods appear to be time consuming and not particularly sensitive and they have been almost totally replaced by molecular methods, especially for diagnostic applications (89).

RT-PCRs amplify portions of viral genome, allowing a more rapid detection. Furthermore its high sensitivity enables viral presence to be revealed for a longer period compared to isolation methods or other detection techniques (51). Several PCR protocols have been described. A PCR based on the highly conserved N gene has proved to be able to detect all four AMPV subtypes (13). Subtype specific PCRs have been suggested targeting more variable genes like G (13, 154) and F (109, 143) for subtypes A and B and M for subtype C (4, 173). Real Time PCR protocols have been also developed, allowing not just a more sensitive viral detection but even viral quantification (41, 95). Multiplex RT-PCR have also been tested, able to detect AMPV and other respiratory viruses such as Influenza, Newcastle Disease and IBV (3, 83, 140).

1.7.2 Indirect Diagnosis

As antibodies against AMPV have been proved to be detectable for at least 89 days in sera after infection (121), serological tests are commonly used to confirm infection, especially in commercial poultry (89). Virus neutralization (VN), indirect immunofluorescence (IIF) (Figure 5), and enzyme-linked

immunosorbent assay (ELISA) tests have all been used. However while the first two are mainly employed for research work, ELISA is now the most common serological AMPV test, due to its sensitivity, specificity and suitability for mass serological screening. Several commercial kits have been developed (49, 89). Performances are highly dependent on the coated antigen: homologus tests have shown a higher efficiency compared to heterologous ones, especially among different subtypes (51, 148, 214). This can give rise to false negatives and the illusion that a vaccine has not 'taken'. Subtype C antibodies are detected very poorly by subtype A and B ELISA, leading to the production of subtype C specific test (55, 138). Finally Blocking ELISAs are available in order to detect antibodies originated from sera of any avian species (33, 218).

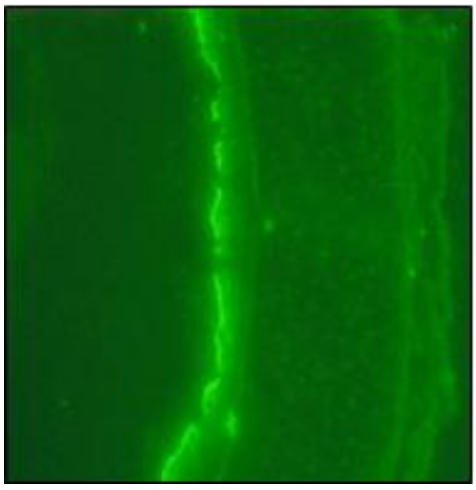

Figure 5: IIF positivity to AMPV on tracheal section

(provided by E. Catelli)

1.8 CONTROL

As specific therapy against AMPV is not available, a preventive approach is critical both in avoiding the infection of birds and in controlling eventual losses caused by the disease. Attention to hygiene and biosecurity practices, ventilation, temperature, density, stress control, disinfection and good management procedures are all critical in reducing symptomatology and mortality (89, 205). Antibiotics can be used to prevent secondary bacterial infection (42, 97).

1.8.1 Vaccination

AMPV infections can be prevented by vaccination. Several vaccines are available and commonly used in commercial birds. Live attenuated vaccines are usually administrated by several methods (intranasal, eye-drop, drinking water or spray) to all bird categories at early stages to prevent the respiratory symptomatology (49, 89). While in broilers one administration seems to be fully protective, in growing turkeys repeated vaccination are required. Laying birds are usually vaccinated prior to the onset of lay by injection of inactivated vaccines, to avoid egg production losses (49, 63). Good cross protection has been reported between A and B subtypes (56, 74, 214). On the other hand, subtype C vaccines do not protect against A and B subgroups (55). Simultaneous vaccination with AMPV and other respiratory viruses (IBV, NDV) is not advised by pharmaceutical companies. However, experimental studies have shown no interference in protection onset (54, 79, 81). *In ovo* administration of live AMPV vaccines has been also tested by different research groups with good results (103, 208, 237). Recombinant and subunit vaccines

have been recently developed and evaluated, however with poor outcomes at the time of writing, compared to conventional vaccines (107, 123, 179, 209).

1.9 REVERSE GENETICS

In 2004 *Naylor et al.* developed the first reverse genetic system for AMPV (158). A full length (FL) cDNA of subtype A was cloned in a plasmid vector by a series of PCR and ligation steps. The plasmid included a kanamicyn-resistant gene, essential in the cloning process, a T7 promoter and *Hepatitis delta virus ribozyme*. (158). N, P, L and Matrix 2 (M2) single genes, lead by a T7 promoter, were also cloned in other plasmids in order to provide the genome with the essential protein to form the RNP complex. Viral rescue was performed on Vero cells, previously infected with a recombinant *Fowlpox virus* expressing the bacteriophage T7 polymerase. This polymerase is able to recognize the T7 promoter inserted in the plasmids and then to initiate transcription directly from them. Therefore the addition of the full length cDNA and the four support protein genes, in the presence of lipofectamine 2000 to allow cDNAs entrance into cells, should lead to the formation of all the RNP components (158). After the complex has been established, genome replication and gene transcription can begin as occur naturally, producing new RNA virions. A similar system, based on the same technical principles, was constructed for subtype C in 2006 in the United States (92). Up to now it has not yet been possible to apply RG system for subtype B.

Since RG has been applied to AMPV (92, 158) several works have investigated the effect of single and multiple genomic mutations (23, 160, 163) but also of gene deletions (133, 243) and insertions (92, 136) on viral biology.

CHAPTER 2

AVIAN METAPNEUMOVIRUS RT-NESTED PCR: A NOVEL FALSE POSITIVE REDUCING INACTIVATED CONTROL VIRUS WITH POTENTIAL APPLICATIONS TO OTHER RNA VIRUSES AND REAL TIME METHODS

TABLE OF CONTENT

2.1 ABSTRACT

Using reverse genetics, an *Avian metapneumovirus* (AMPV) was modified for use as a positive control for validating all stages of a popular established RT-nested PCR, used in the detection of the two major AMPV subtypes (A and B). Resultant amplicons were of increased size and clearly distinguishable from those arising from unmodified virus, thus allowing false positive bands, due to control virus contamination of test samples, to be readily identified. Absorption of the control virus onto filter paper and subsequent microwave irradiation removed all infectivity while its function as an efficient RT-nested- PCR template was unaffected. Identical amplicons were produced after storage for one year. The modified virus is likely to have application as an internal standard as well as in real times methods. Additions to AMPV of RNA from other RNA viruses, including hazardous examples such HIV and Influenza, are likely to yield similar safe RT-PCR controls.

2.2 INTRODUCTION

Avian metapneumovirus (AMPV) causes major disease in poultry in most of the world and its detection and characterization has assisted (84, 98, 145, 234), and continues to assist (34, 39), in vaccine selection and disease control. RT-PCR is a powerful technique for detecting many viruses through the presence viral RNA in diagnostic samples. For *Avian metapneumovirus* (AMPV) tests have been reported (38, 110, 154, 167) which offer a sensitivity generally similar to, or greater than, classical virus isolation while additionally extending detection beyond loss of replicative viability (154), as well as frequently informing of virus origins through RNA sequence analysis (31, 34). Furthermore this sequence analysis avoids the need for secondary virus identification as often required in isolation techniques (17).

For PCR tests a positive control DNA sample generally accompanies sample tests to ensure the detection system is functional. However its cross contamination of test samples can lead to false positive readings (128). For RT-PCR tests, a control RNA is equally necessary and generally takes the form of an established control virus, but again its cross contamination of samples can be confused with true positive detections. This paper describes the construction and testing of a novel positive control AMPV able to identify such cross contamination events by producing amplicons of increased sizes, readily distinguishable from field virus amplicons on agarose gels, when used in conjunction with an established G gene based RT-nested-PCR method, (154).

The established RT-nested-PCR method, distinguishing between AMPV subtypes A and B, has been in common use since 1995 and is outlined in Figure 6. Briefly, genomic sense primer G6- primes from antigenome sense RNA to yield a DNA copy. This is amplified using the same primer together with

antigenomic sense primer G1+. The resultant amplicon is further amplified using subtype specific PCRs whereby common primer G5- is paired with G8+A for subtype A specificity and G9+B for subtype B specificity. Subtype A and B viruses result in bands of 268 bp and 361 bp respectively and are readily distinguished on agarose gels.

More recently, the presence of the AMPV G gene was demonstrated unnecessary for AMPV virus growth in Vero cells (133, 158, 160) and this made possible the generation of viruses with dysfunctional or absent G genes by reverse genetics (158, 161). This also led to the possibility of generating viable diagnostic RT-PCR control viruses containing major G gene modifications. The current study describes the construction and testing of a novel subtype A Vero-grown RT-nested-PCR positive control virus possessing two G gene modifications, with one conferring subtype B primer specificity and the other resulting in RT-nested PCR amplicons of increased size. Subsequently, the virus was absorbed onto filter paper and inactivated by microwave radiation to yield a stable, non-infectious standardized control sample.

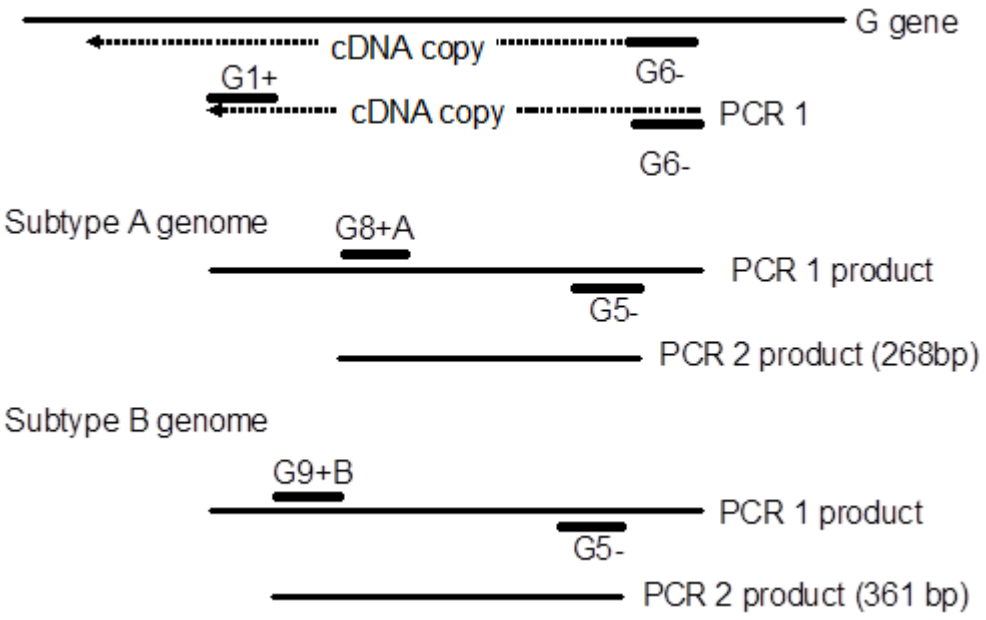

Figure 6: Schematic diagram showing the usage of oligonucleotide primers in the established RT-nested-PCR for detecting RNA from either subtype A or B AMPVs.

2.3 MATERIALS AND METHODS

Establishment of an AMPV control virus. A full length (FL) cloned viral copy of a subtype A AMPV LAH A (158) was modified in two stages; initially to add B type specificity, then a spacer was added to yield amplicons of increased size. Modifications are outlined in Figure 7 and details are given below.

AMPV full length copy: addition of G9+B sequence. A full-length DNA copy of AMPV LAH A was modified by site directed mutagenesis to introduce the subtype B G gene primer sequence, G9+B (Table 1) at the equivalent position in the subtype A G gene, as shown in Figure 7 (a). Briefly primer Ga-G9+b contained the sequence of G9+B flanked by subtype A sequence and Ga-G9-b contained the exact reverse compliment sequence. Annealing of these primers to the cloned LAH A copy and extension using pfu turbo, led to a nicked modified full length copy which was used to transform Invitrogen STB12 cells as previously described (22, 43, 161). Colonies were screened for the presence of the correct sequence by PCR using G9+B and G5- primers.

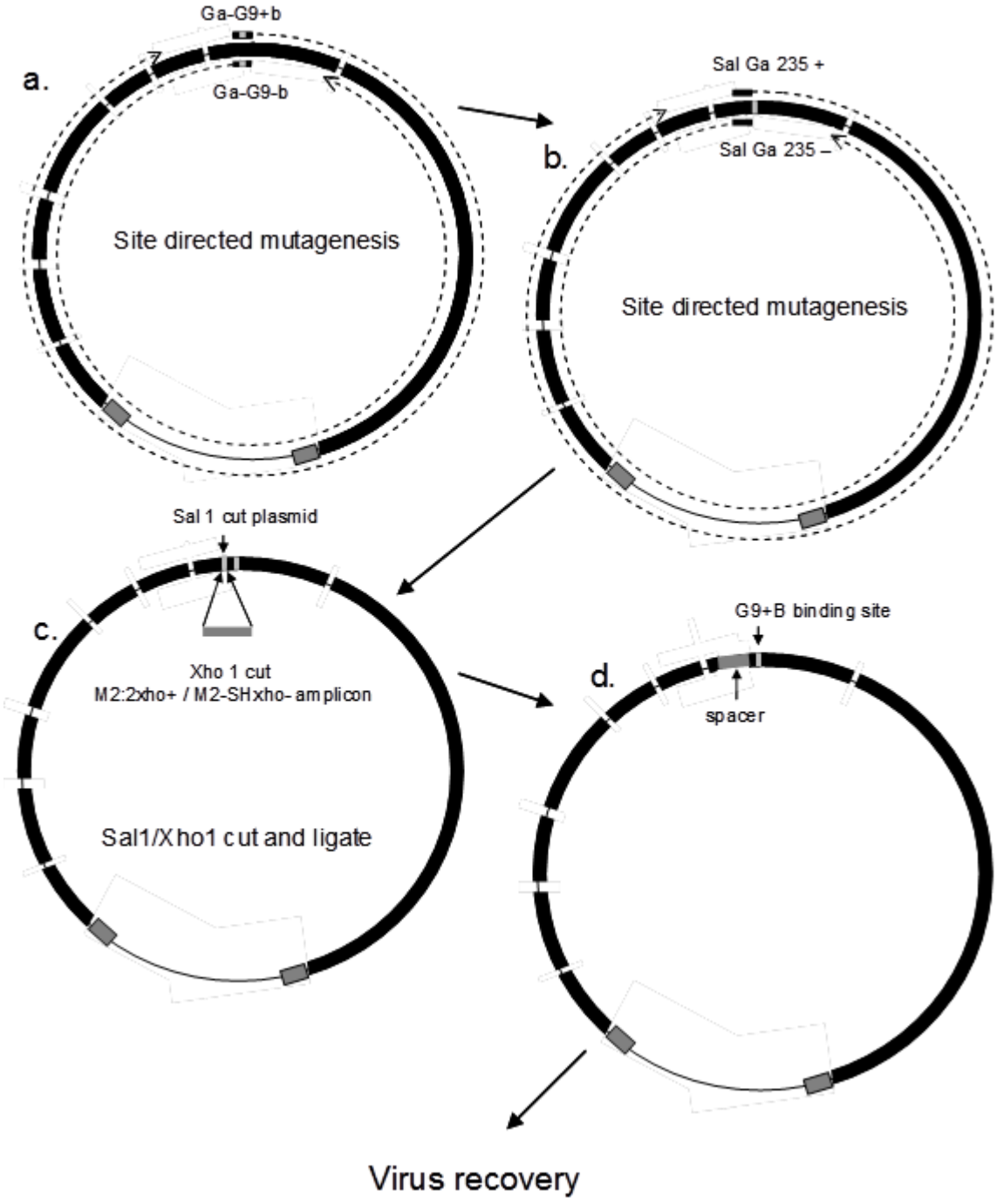

Figure 7: Outline of the stages involved in modification of the LAH A full length copy

Table 1: Oligonucleotide primers used in the RT-nested-PCR or construction of the modified full length cDNA

PRIMER	SEQUENCES
G 1 +	gggacaagtatctctatg
G 6 -	ctgacaaattggtcctgatt
G 8 + A	cactcactgttagcgtcata
G 9 + B	tagtcctcaagcaagtcctc
G 5 -	caaagagccaataagccca
M2.2 Xho +	gtgcaatgctcgaggattgtgtatgg
M2-SH Xho -	ccatacacaatcctcgagcattgcac
Sal Ga 235 -	ggctgctcctgggtgggtcgaccaccaatctctatctcctcc
Sal Ga 235 +	ggaggagatagagattggtgtcgacccacccaggagcagcc
Ga-G9-b	gccaatatgtacctcctcccgaggacttgcttgaggactactgcagtttgatatgc
Ga-G9+b	gcatatcaaactgcagtagtcctcaagcaagtcctcgggaggaggtacatattggc

AMPV full length copy: insertion of spacer sequence. To increase RT-nested-PCR amplicon sizes, a spacer was added within the amplified region between binding sites for the opposing primer pairs, as shown in Figure 7 (b). A section of 216 bp was amplified from the AMPV M2 gene using M2:2xho+ and M2-SHxho- primers which added Xho1 sites close to each end. A Sal1 site was introduced to the FL copy between G8+A and G5-, using Sal Ga 235 – and Sal Ga 235 + oligos. After digestion of the plasmid and 216 bp amplicon with Sal 1 and Xho1 respectively, both were ligated together (Figure 2 (c)) in the presence of Sal 1 and the ligation mixture was used to transform Invitrogen STB12 competent cells. Following colony growth and screening using a junction PCR, one was grown, from which modified plasmid was prepared. Prior to further development, PCRs using nested oligos G8+A, G9+B and G5- were performed.

Control Virus Recovery. Virus recovery was attempted as previously described (158). Briefly, the modified full length copy and three plasmids expressing AMPV N, P and L genes, all with upstream T7 promoter sequences,

were used to transfect Vero cells previously infected with a fowlpox recombinant virus expressing T7 polymerase (20). The cells were incubated for 6 days and material serially passaged twice in fresh Vero cells. From the first passage onwards, cell sheets were examined daily for cytopathic effects (CPE). Freeze thawed lysates were further passaged in fresh Vero monolayers.

Virus irradiation and storage. The Vero cell lysates containing the modified virus were freeze-thawed twice then absorbed onto Whatman no 1 filter paper. After air-drying, this was microwave irradiated at maximum power (900 W) for two minutes using a protocol previously demonstrated to inactivate virus, but not inhibit the detection of viral RNA by RT-PCR (71). The filter papers were cut into 3 x 0.5 cm pieces and stored in 1.5 ml flip top tubes at 4°C. To assess virus inactivation, treated papers were immediately soaked in Vero cell culture medium, vortex mixed then resultant liquid was used to inoculate Vero cell monolayers.

Testing of the modified virus as an RT-nested-PCR control. The control virus was tested alongside subtype A (UK3B (60) and subtype B virus test viruses (Merial Aviffa). The details and sizes of the expected RT and PCR products for the newly generated control virus are given in Figure 8. Immobilised, microwave irradiated control virus was identically tested as was the same material after storage for one year.

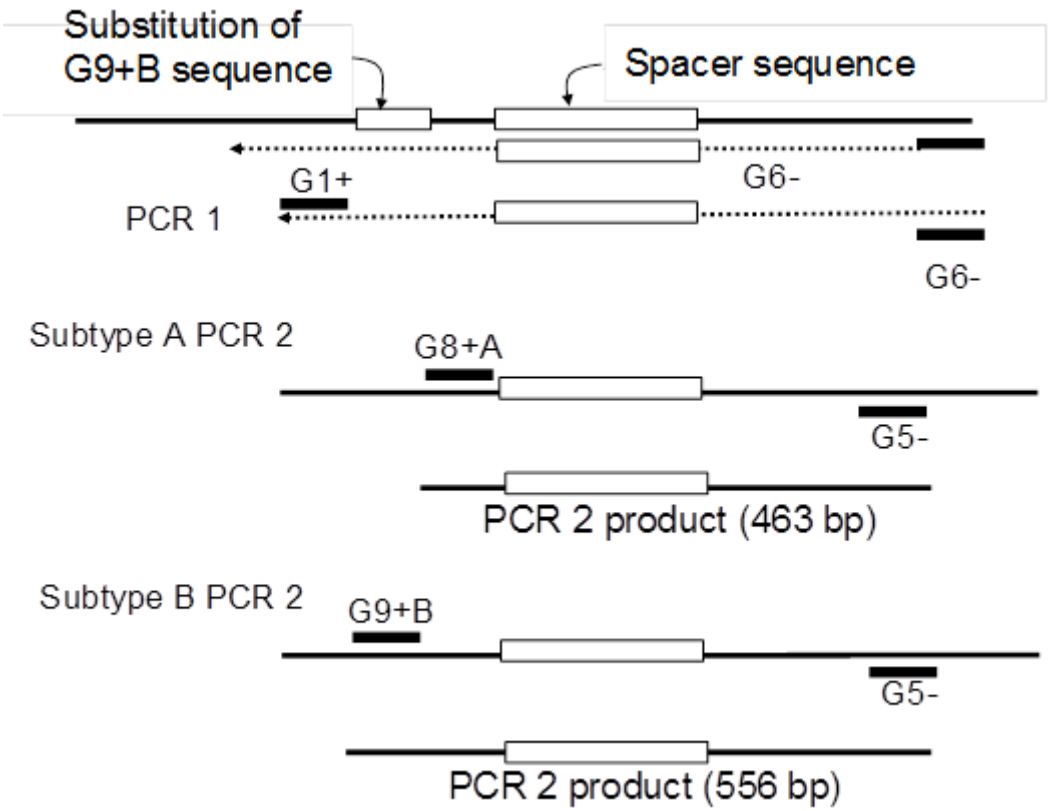

Figure 8: Outline of the RT-nested-PCR stages and sizes when using the modified control virus and standard test components

2.4 RESULTS

Modification of AMPV LAH A FL clone. Using the G9+B/G5- primed PCR, the modified FL clone yielded a product of the expected size of 360 bp on a 2% agarose gel, thereby confirming that the G9+B sequence had been added as designed. Following addition and cloning of the Xho1 cut 216 bp spacer region, PCR tests using G8+A/G5- and G9+B/G5- primer combinations produced products with the expected sizes of 463 and 556 bp respectively, thereby confirming that the FL copy had been successfully modified (data not shown).

Recovery of modified virus. Seven days after transfection of Vero cells with the modified cloned FL and support plasmids, material was freeze-thawed.

Four days after inoculation of fresh Vero monolayers, CPE typical of AMPV infection was readily observed by low power microscopy.

RT-nested-PCR control of recovered virus. Analysis of RT-nested-PCR products on a 2% agarose gel showed bands of 268 bp for subtype A virus, 361 bp for subtype B virus, no band for the negative control and bands of 463 and 556 for the inactivated positive control virus. The results are shown in Figure 9.

Cell culture of microwave irradiated virus. CPE was not observed in Vero cell cultures inoculated with liquid collected from soaked microwave irradiated material. Equally no CPE was seen after a further passage in fresh Vero cells.

RT-nested-PCR control of microwave irradiated virus. Results were identical to those for viable virus (data not shown)**.**

Stability of microwave irradiated virus. When the RT-nested-PCR was performed on material stored for one year at $4^{o}C$, results were identical to those seen one year earlier (data not shown).

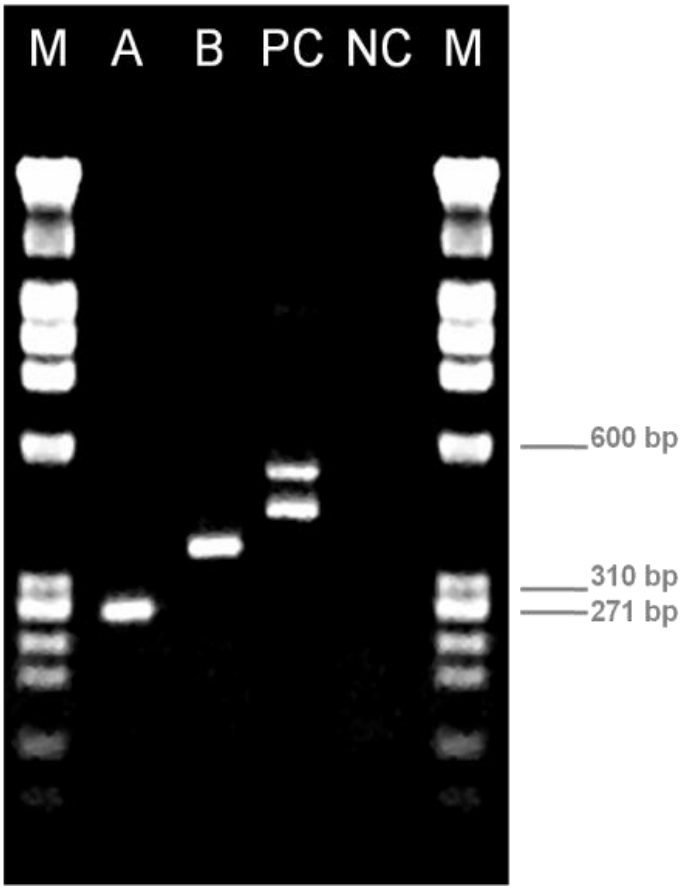

Figure 9: 2% agarose gel of the RT-nested-PCR G gene products with templates using UK3B (A), Merial Aviffa (B) and modified control virus (PC). Also water control (NC) and size markers (M)

2.5 DISCUSSION

RT-nested-PCR is a flexible virus detection technique of similar sensitivity to efficient isolation techniques while affording the advantage that only the intact targeted RNA is required for positive detections. However, there is also greater risk of test positives due to contamination with residual environmental nucleic acids, whereas in contrast, infection of culture based detection systems with viable remnants of environmental viral contaminants would be unlikely. The necessary inclusion of positive control standards in detection runs adds to this risk (128) and its relative impact is greater if the

majority of test samples are negative, due to risk of contamination from the control virus. Nevertheless, positive control standards are generally included because of the need to distinguish between true detection negatives and those arising from test system malfunction. The novel control virus made and tested in this study removes the need to choose between conflicting demands. As for conventional AMPV control standards, the presence of correct bands confirms that all stages of the detection system, RNA extraction, reverse transcription and PCRs, are functional but in addition, the increased size of amplicons immediately identifies instances where sample test positives result from contamination with the novel control.

An alternative to the described strategy might be T7 driven in vitro transcripts generated from suitably constructed plasmids. However this would have several disadvantages, one of which being that transcripts would not act as controls of RNA extraction from virus. Another consideration would be that remnants of the plasmid DNA would need to be scrupulously removed otherwise its presence would invalidate the check of reverse transcription. Finally it is not clear whether stored immobilised naked RNA would share similar stability because in the case of the described control virus, the RNA is likely to have been stabilised by other viral components including the ribonuclear proteins.

A potential problem with adopting such a live control virus in some regions might be its genetically modified nature. However, the microwaved-irradiated virus was shown to work equally well and Vero cell culture was used to confirm that all virus infectivity had been lost. Furthermore, the inactivated virus was found to remain fully functional as a control after a year of refrigerator storage. Hence a control has been developed which does not present any infection risks so can be used safely in all laboratory situations.

The control virus described might also find useful application as an internal control. In principle the control could be simply added to all test unknowns. Both normal and larger control virus induced amplicons would be seen for positive samples, while test samples lacking AMPV target RNA would generate only the larger amplicons. However before being used in this manner, the test would need to be validated to ensure that competition or other unforeseen events did not interfere with the function of either sample or control RT-nested-PCR tests. This is likely to be an area of further investigation in our laboratory.

The methodology is likely to be similarly useful for preparation of positive controls for the many RT-PCR tests used for detection of other RNA viruses, both avian (38, 85, 204) and non-avian (10, 27, 72). The addition of a complete foreign gene to subtype A AMPV has already been demonstrated (137) hence it will be a routine matter to similarly add sequence from viruses such as those listed, irrespective of their role in host viruses. Again the foreign virus sequence would need modification to enable amplicon size differentiation but problems of its incorporation into AMPV anywhere within the regions coding for the nonessential SH or G genes (158) would not be expected to curtail virus replication. Furthermore, while the inserted small RNA sections would be highly unlikely to produce hazardous viruses, this risk would be entirely eliminated using the described microwave irradiation procedure. This would make the approach very suitable for generating controls for frequently used RT-PCR tests detecting hazardous viruses, including influenza and HIV.

The use of the modified virus as a positive control could also find application in real time RT-PCR. For detections utilising Taq polymerase catalysed hydrolysis of an annealed fluorescent probe, the application is not immediately obvious because the probe should bind to equally well to normal or larger test amplicons. However the larger size of control virus amplicons

would generally result in higher melting temperatures and this difference could be detected in suitable real time machines. A similar approach could also be taken using sybr green based PCR tests. In both cases, the design of modified viruses would need to be directed towards inserting RNA within the test amplified regions so as to give the necessary melting temperature differences, while not significantly compromising the efficiency of the given RT-PCR test.

In summary, a modified AMPV has been generated for use as a positive control in an established RT-nested PCR which, after microwave inactivation for safety considerations and fridge storage for long-term convenience, confirms test function at all test stages from initial RNA extraction from virus to final visualization of bands on gels. It has the further potential to be used as an internal standard and related modifications could yield real time RT-PCR applications. The main application may lie with other RNA viruses, especially those presenting a significant safety risk. Insertion of RNA from those viruses into AMPV should result in safe controls for a large range of hazardous RNA viruses including HIV and influenza.

CHAPTER 3

AN INVESTIGATION INTO VECTORING PROPERTIES OF *AVIAN METAPNEUMOVIRUS*

TABLE OF CONTENT

3.1 ABSTRACT

Seven different AMPV cDNAs, each one in a different intergenic region, were modified in order to accept and express a reporter gene. A cassette was firstly introduced adding a restriction enzyme site, for actual genic sequence insertion, and transcriptional start and stop signals, essentials for gene transcription. GFP was then inserted in all constructs and after viral rescue, seven viruses carrying the reporter gene were obtained. Expression was assessed firstly by fluorescence observation under UV microscope and later using a specific GFP ELISA, able to quantify the amount of protein produced. Viruses were titrated using both CPE endpoints on Vero cells and real time RT-PCR, to calculate the amount of protein produced by a single infectious viral dose, and sequenced to detect possible mutations occurring during viral passages. Results showed AMPV to be able to accept and stably express GFP in all the positions. All recombinants were able to reach high titers with the only exception of the one expressing GFP between N and P. Expression proved to be highest when GFP was inserted near the 3' end (between N and P) as expected according to the transcriptional model for non-segmented negative stranded viruses. Surprisingly even the recombinant with the insert in the SH-G position showed high production of the reporter gene. Conversely GFP levels were very low in the middle positions.

3.2 INTRODUCTION

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 not just the effect of specific mutations on viral biology but also to perform major changes such as deleting or adding genes (47, 48, 229). Since RG has been established, several NNS RNA recombinant viruses expressing foreign genes have been constructed in order to develop improved or multivalent vaccines (164, 189). These viruses have been shown to be suitable candidate as vectors: (I) they do not replicate through DNA intermediates, so integration of the foreign gene into the host genome is very unlikely; (II) recombination is extremely rare; (III) they have a simple genome organization, involving only 5-11 proteins and genes are usually not overlapping, making manipulations easier; (IV) they grow to high titres and express high levels of proteins; (V) they commonly induce strong humoral and cellular immune responses (48, 189, 229) and (VI) they have been proven to accept and stably express foreign genes without mutations incurring over several passages (147, 192). *Avian metapneumovirus* (AMPV) is a member of this order, belonging to the family *Paramyxoviridae*, subfamily *Pneumovirinae*. It transcripts for 8 different genes, namely N, P, M, F, M2, SH, G and L (70). AMPV affects mainly turkeys and chickens causing respiratory infections and reproductive effects on laying birds, such as breeders and laying hens and secondary infections exacerbate the effect of the primary virus infection (50). In 2004 the first RG system was developed for AMPV (158) and since then several

studies have investigated the effects of single and multiple mutations or even whole gene deletions (23, 44, 92, 133, 158, 163). A few reports have shown the possibility to add and express foreign genes in the viral genome (92, 136), suggesting the possibility of using AMPV as a vector for a multivalent vaccine. However, during the construction of recombinant viruses several technical questions might arise regarding for example the insertion position of the new gene. Current transcriptional models for NNS RNA viruses propose that viral polymerase should enter the genome only at position 1 at the 3' leader end, transcribing all genes obligatorily in a sequential and polar manner (from 3' to 5'). Along the RNA, polymerase encounters specific gene start and stop signals. The latter ones seem to facilitate the fall off of a certain amount of polymerase molecules from the genome, while the rest of them carry on in the transcription process. This results in a quantity of polymerases able to move toward 5' end, which gradually decrease at every gene junction. Levels of gene expression are then regulated primarily by the position of each gene relative to the 3' end, meaning upstream genes are more easily transcribed than downstream genes (233). According to this, it would be preferred to add a foreign gene at the proximal 3' position, as it would result in higher expression of the insert and, thinking to a future vaccine use, this might be critical for viral immunogenic activity in birds. However a previous study using *Vescicular stomatitis virus* (VSV) has shown that genes added in a very early position can be a problem for virus viability (232).

In order to try to understand, which position is more likely to give best expression performances for AMPV without penalizing viability, seven different viruses were constructed, expressing green fluorescent protein (GFP) in different intergenic regions (IGR). GFP, surrounded by new gene

start and new gene stop sequences, was introduced at every IGR in AMPV full length cDNAs, which were then rescued according to *Naylor et al.* (158). Recovered viruses were passaged three times on Vero cells and then titrated by observing cythopatic effect (CPE) endpoints as an indicator of viral viability in vitro. Inserted gene expression was firstly assessed by observing the fluorescence under UV microscope and then quantified using a specific GFP ELISA. ELISA results were then compared to viral titres in order to calculate the protein expression quantity per single infectious dose. However, as the previous titration method is based on capacity of the virus to infect cells, all viruses were also titrated with real time RT-PCR for a better accuracy (184). Real time RT-PCR directly detects viral RNA, revealing the presence of viral particles which have a lack of infectivity, but which can still transcribe genes (184). This appears critical in avoiding under or over estimation of viral titres and then of GFP expression. Specific messenger RNA (mRNA) RT-PCRs (23) for every upstream gene to the insertion point were also performed in order to evaluate the functional gene stop. Polymerase failure in stopping at the signal can lead to the production of dicistronic messanger RNA, resulting in a lack of expression of the insert (233).

3.3 MATERIALS AND METHODS

Standard cassette insertion, allowing gene expression. Seven full length AMPV cDNAs of an A derived strain (158) were modified to add a cassette in each one of the seven IGR present in the genome. The cassette was designed and ordered. It comprised two annealed complementary primers (Cas

+ and Cas neg) and included a transcriptional start (GGGACAAGT), a Sal I restriction endonuclease site (GTCGAC) and a transcriptional stop (AGTCAATAAAAAA) (Figure 10). Xho I restriction endonuclease sites were previously inserted in the genome by site-directed mutagenesis (SDM) to allow insertion. Ligation of the cassette was performed in the presence of Xho I. Primers used for this aim are shown in Table 2.

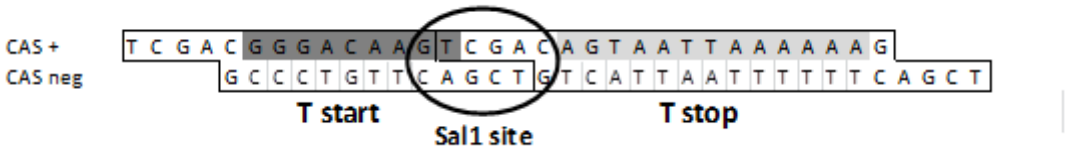

Figure 10: Cassette showing transcriptional start (T start), restriction endonuclease site (Sal1 site) and transcriptional stop (T stop).

GFP insertion. GFP gene was amplified using primers GFP ins + and GFP ins neg which added XhoI sites to both ends. After Xho I digestion of the amplicons and Sal I digestion of the cassetted AMPV cDNAs, these were ligated together in the presence of both enzymes. Figure 11 summarizes the modifications made in the IGR. After cloning into stb12 cells, colonies containing DNA with correct gene orientation and sequence were selected. The modified constructs were then sequenced to exclude possible mutations, which may have occurred during these processes. Seven cDNAs were selected, each one with GFP in different IGR (Figure 12).

Viral rescue. GFP AMPV cDNAs were rescued as described by Naylor et al in 2004. Modified full length copy and four plasmids expressing AMPV N, P, M2 and L genes, all with upstream T7 promoter sequences, were used to transfect Vero cells previously infected with a fowlpox recombinant virus expressing T7 polymerase (20). The transfected cells were incubated for 6 days and the recombinant viruses were serially passaged in fresh Vero cells.

Table 2: Oligonucleotide sequences of premises used in the study

Primers Name	Primers (5'... 3')	Function
IGR-Xho-NP +	CAAATTTGAGTAATTAAAAACTCGAGGGACAAGTAACAATG	Insertion XhoI site betw een N and P genes
IGR-Xho-NP neg	CATTGTTACTTGTCCCTCGAGTTTTTAATTACTCAAATTTG	Insertion XhoI site betw een N and P genes
IGR-Xho-PM +	GATCTGTAGTTATGAAAAACTCGAGGGACAAGTCAAAATGGAG	Insertion XhoI site betw een P and M genes
IGR-Xho-PM neg	CTCCATTTTGACTTGTCCCTCGAGTTTTTCATAACTACAGATC	Insertion XhoI site betw een P and M genes
IGR-Xho-FM2 +	CAGTTAAGTTATTTAAAACTCGAGGGACAAGTGAAGATGTC	Insertion XhoI site betw een F and M2 genes
IGR-Xho-FM2 neg	GACATCTTCACTTGTCCCTCGAGTTTTAAATAACTTAACTG	Insertion XhoI site betw een F and M2 genes
IGR-Xho-M2SH +	GTTAATTAAAACCACTCGAGCTATAAGGCCAATAAAGG	Insertion XhoI site betw een M2 and SH genes
IGR-Xho-M2SH neg	CCTTTATTGGCCTTATAGCTCGAGTGGTTTTAATTAAC	Insertion XhoI site betw een M2 and SH genes
IGR-Xho-SHG +	GTATTATTTAATTAAAAAAAACTCGAGGGACAAGTATCTCAATG	Insertion XhoI site betw een SH and G genes
IGR-Xho-SHG neg	CATTGAGATACTTGTCCCTCGAGTTTTTTTTAATTAAATAATAC	Insertion XhoI site betw een SH and G genes
IGR-Xho-GL +	GTCTAAAACAATTAAACTCGAGAAAAACAAGGACCAATATG	Insertion XhoI site betw een G and L genes
IGR-Xho-GL neg	CATATTGGTCCTTGTTTTTCTCGAGTTTAATTGTTTTAGAC	Insertion XhoI site betw een G and L genes
MF Xho I +	GTTATAGTCAATAAAAAATTCTCGAGGGACAAGTAGGATGGATGTA	Insertion XhoI site betw een M and F genes
MF Xho I neg	CAGATTCTTACATCCATCCTACTTGTCCCTCGAGAATTTTTTATTG	Insertion XhoI site betw een M and F genes
Cas +	TCGACGGGACAAGTCGACAGTAATTAAAAAAG	Cassette construction
Cas neg	TCGACTTTTTTAATTACTGTCGACTTGTCCCG	Cassette construction
GFP ins +	GGGACCTCGAGTATGGTGAGCAAGGGCGAGGAGC	GFP amplification
GFP ins neg	CCACTCCTCGAGATTTTACTTGTACAGCTCGTCC	GFP amplification
N1+	CAATATAATGTTGGGCCATG	N gene mRNA RT-PCR
P1+	GCAATGATAGGGATGAGA	P gene mRNA RT-PCR
M8+	GAAGCTGCAATAAGTGGGGAAG	M gene mRNA RT-PCR
F8+	CCCTGAGGATCAGTTCAATGTTGC	F gene mRNA RT-PCR
M2 MID FOR	CCAGAGATTCAATGCTTGAAGACCC	M2 gene mRNA RT-PCR
SH70 +	GGACAGTGATCAAGTAAAGGTGC	SH gene mRNA RT-PCR
G7+	GAAAAGACATTCAGTACATAC	G gene mRNA RT-PCR

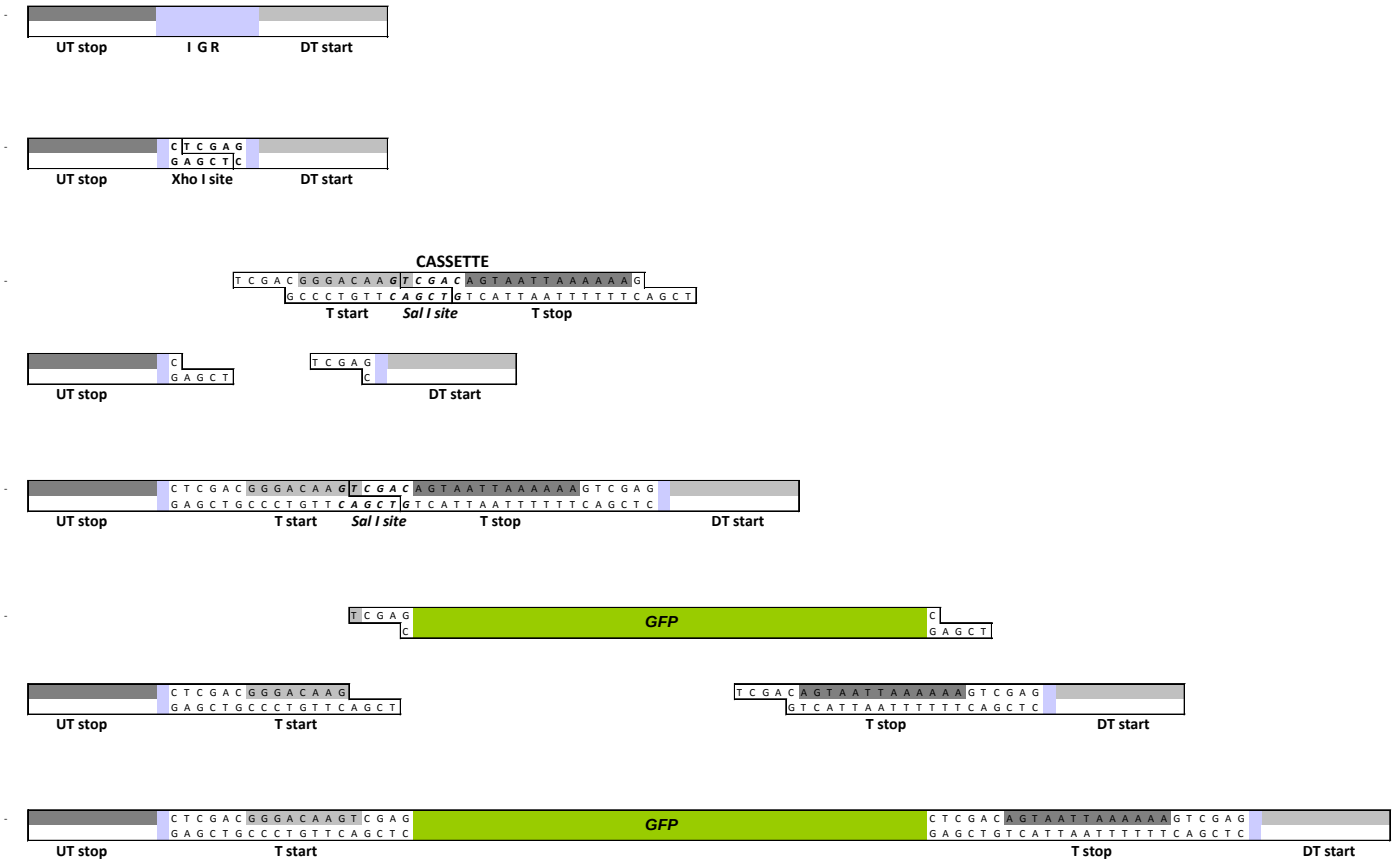

Figure 11: Schematic representation of the strategy used for adding genes to cloned AMPV genome copies. An Xho1 RE site, then standardized cassette and finally chosen gene for expression (illustrated by GFP) were added to intergenic regions (IGR) between upstream gene transcriptional stops (UT stop) and the downstream gene transcriptional starts (DT start).

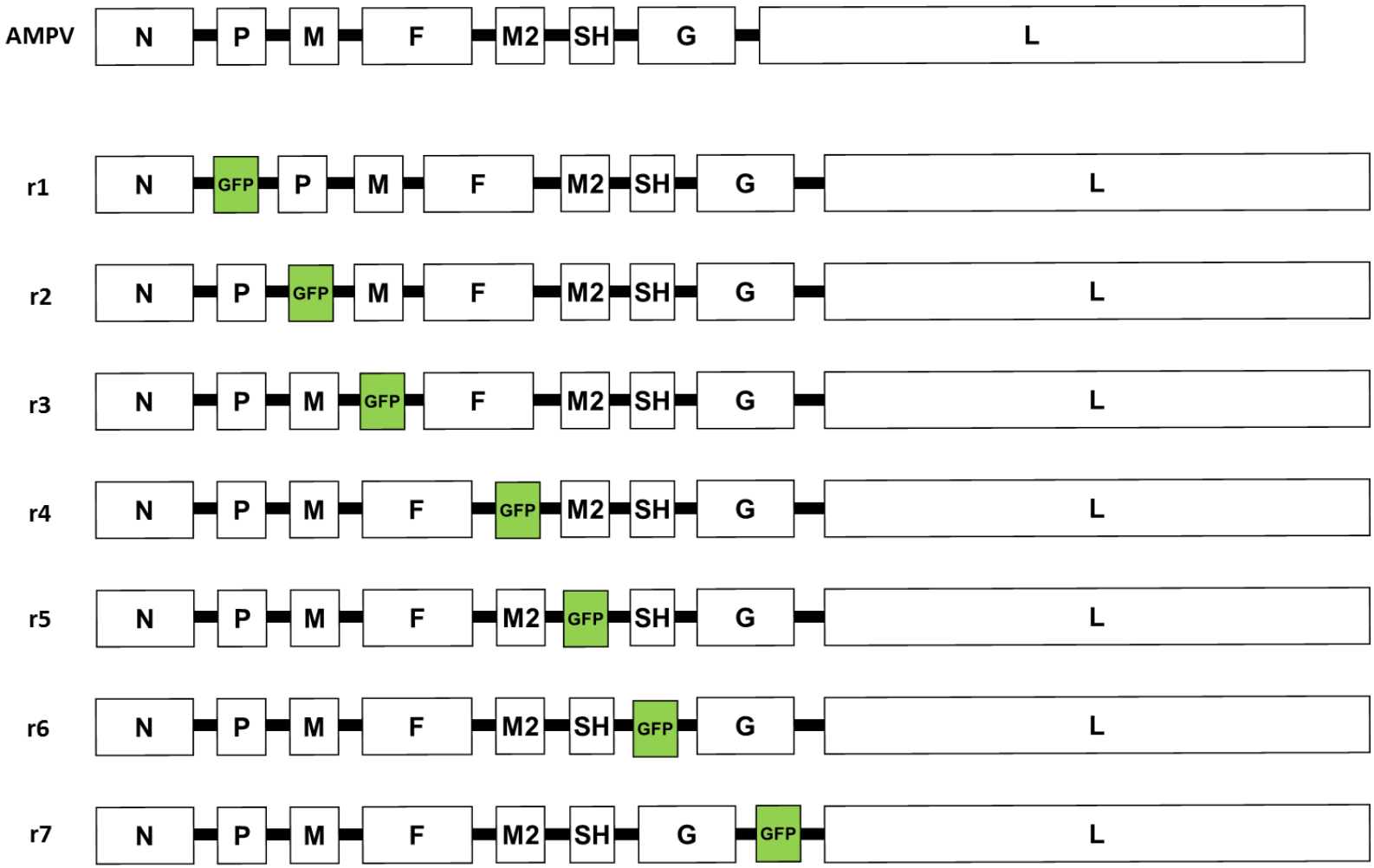

Figure 12: Seven cDNAs were constructed, each one including GFP in a different intergenic position

From the first passage onwards, cell sheets were examined daily for cytopathic effect (CPE). Freeze-thawed lysate was further passaged three times in fresh Vero monolayers.

GFP expression. As GFP has the capacity to exhibit bright green fluorescence when exposed to light in the ultraviolet range, infected cell monolayer sheet were observed under UV optical microscope to prove foreign protein expression.

Viral titration. Viruses were titrated using two different methods. Titration based on CPE endpoints in Vero cells was performed and titres calculated according to *Reed and Muench* (183). Real time RT-PCR specific for AMPV (41) was also used and the obtained data were compared to AMPV standard curves, enabling titres calculation.

Sequencing. Viruses were sequenced in order to check possible mutations occurring particularly in GFP or in the transcriptional cassette during serial passages.

GFP ELISA. Quantification was carried out using a commercial ELISA kit (CELL BIOLABS, INC.) for detection of GFP in cell or tissue samples for all seven viruses. Protein quantities were determined by comparing resultant adsorbance with GFP standards values. Total GFP quantities for each virus were then compared to viral titres to calculate the amount of GFP (pg) produced by a single viral infectious dose.

Upstream gene mRNA RT-PCR. mRNAs were amplified using 3' RACE technique described by *Sambrook and Russell* (188) and adapted to AMPV by Brown et al. (23). RT-PCR was performed to generate N gene mRNA in virus r1, and mRNA for genes P, M, F, M2, SH, and G in virus r2, r3, r4, r5, r6 and r7 respectively (23).

Statistical Analysis. Data normality was assessed using the Kolmogorov Smirnov non parametric test. The one sample T-test was used to test if each one of the modified virus titres was statistically different from the others considered as a unique population. This analysis was performed excluding the most extreme values of the series. The test enabled us to understand from a mathematical point of view, if errors occurred during viral titrations. The relation between virus titres in Vero cells and between pg of GFP per Vero cells infectious dose and pg of GFP per real time RT-PCR infectious dose was investigated using the Pearson's correlation coefficients, which appeared the most appropriate test for the purpose. A probability of $p < 0.01$ was considered statistically significant. All statistical analyses were performed using SPSS for Windows Rel. 12.0.0. 2003 (SPSS Inc., Chicago - IL).

3.4 RESULTS

Recombinant GFP AMPVs. First cassettes and then GFP genes were successfully inserted in each of the different AMPV cDNAs. Seven recombinant viruses, each one expressing GFP in a specific IGR, were rescued on Vero cells, all showing typical AMPV cythopathic effect (CPE). CPE was diffuse in all cell monolayers, excluding the one infected with r1, where only sporadic CPE was observed.

UV microscopy observation. All recombinants produced strong fluorescence when observed under a UV microscope, proving the presence of GFP (Figure 13).

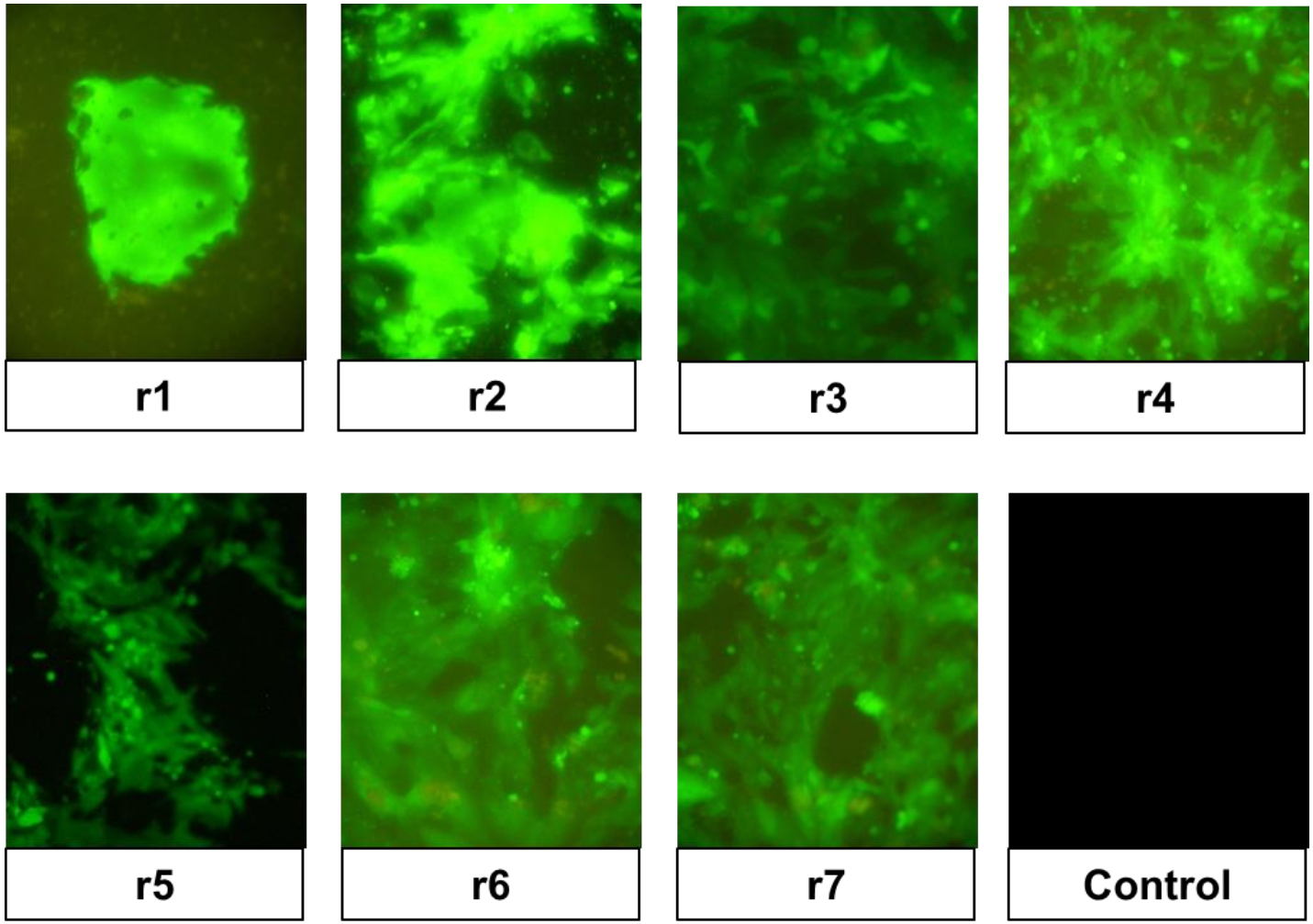

Figure 13: GFP expression observed under UV microscope by the different recombinants. A negative AMPV control was also performed

Viral titration. With the only exception of r1, all viruses showed high titre levels of between 4 and 5 $\log_{10}$ $TCID_{50}$ per ml on Vero cells and between 5 and 6 $\log_{10}$ copies per ml with real time RT-PCR (Table 3). In both methods, the titres of r1 were less than 3 $\log_{10}$ per ml, and proved to be significantly lower ($p<0,01$ and $p<0,01$) than the other viruses. Apart from r1, the results for the two titration methods shared high correlation ($p<0,01$).

Table 3: Viral titres calculated using cell culture or real time RT-PCR

VIRUS	Titrations in Vero cells ($\log_{10}$ $TCID_{50}$ per ml)	Titrations by real time RT-PCR ($\log_{10}$ per ml)
r1	2,14	2,99
r2	4,1	5,7
r3	5	5,55
r4	4,5	5,34
r5	4,85	5,35
r6	4,2	5,04
r7	4,43	5,13

Sequencing. No mutations were detected after 3 passages in any of the viruses, proving the high viral stability despite the extra gene insertion.

GFP ELISA. Figures 14 and 15 show GFP expression in picograms per infectious dose of virus, using Vero cell and real time RT-PCR titrations respectively. GFP expression was highest when GFP was inserted near the 3' (r1) as expected ($p<0,01$ and $p<0,01$), but surprisingly even r6 showed a high expression of the reporter gene ($p<0,01$ and $p<0,01$). GFP levels appeared instead to be very low in the middle positions (r3, r4, r5) and at the very end

(r7). The very high r2 GFP level in Figure 14 compared to the corresponding one in Figure 15 suggests a strong underestimation of the real viral titres by Vero cell titration. This is also confirmed by looking at the statistical correlation between GFP Vero cells and GFP real time RT-PCR when r2 values are not considered (Figure 16).

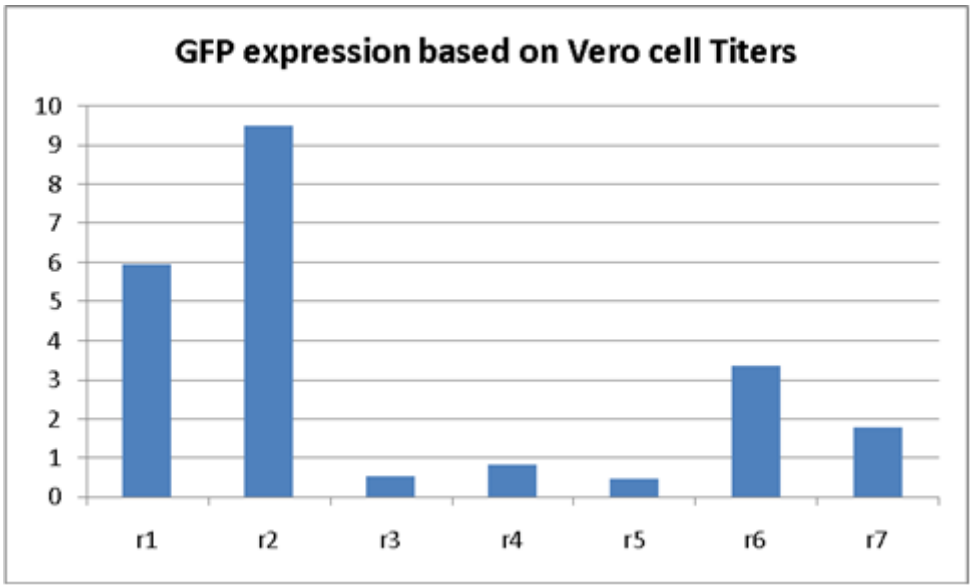

Figure 14: Amount of GFP protein (pg) per single infectious dose based on Vero cell titration

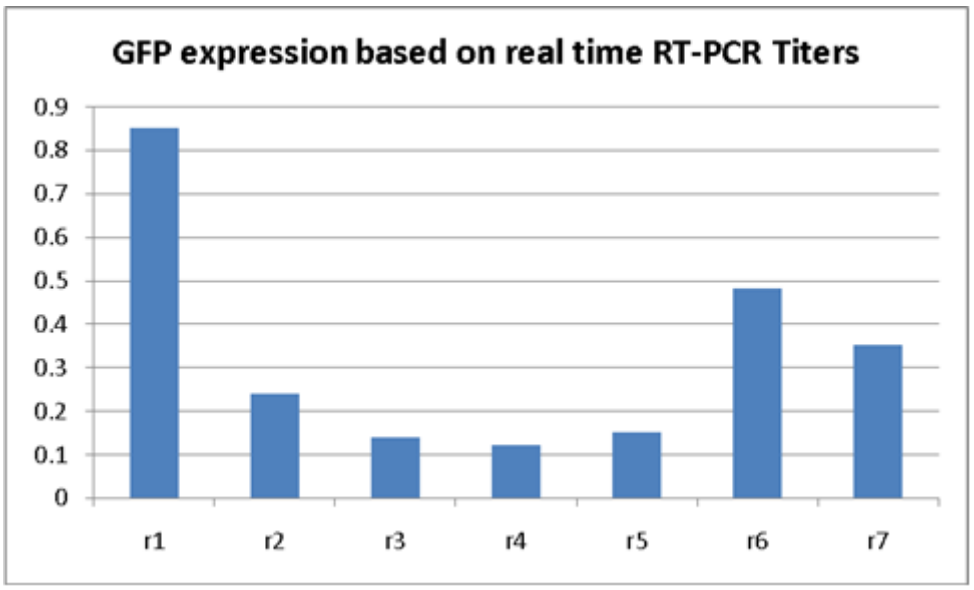

Figure 15: Amount of GFP protein (pg) per single copy based on real time RT-PCR titration

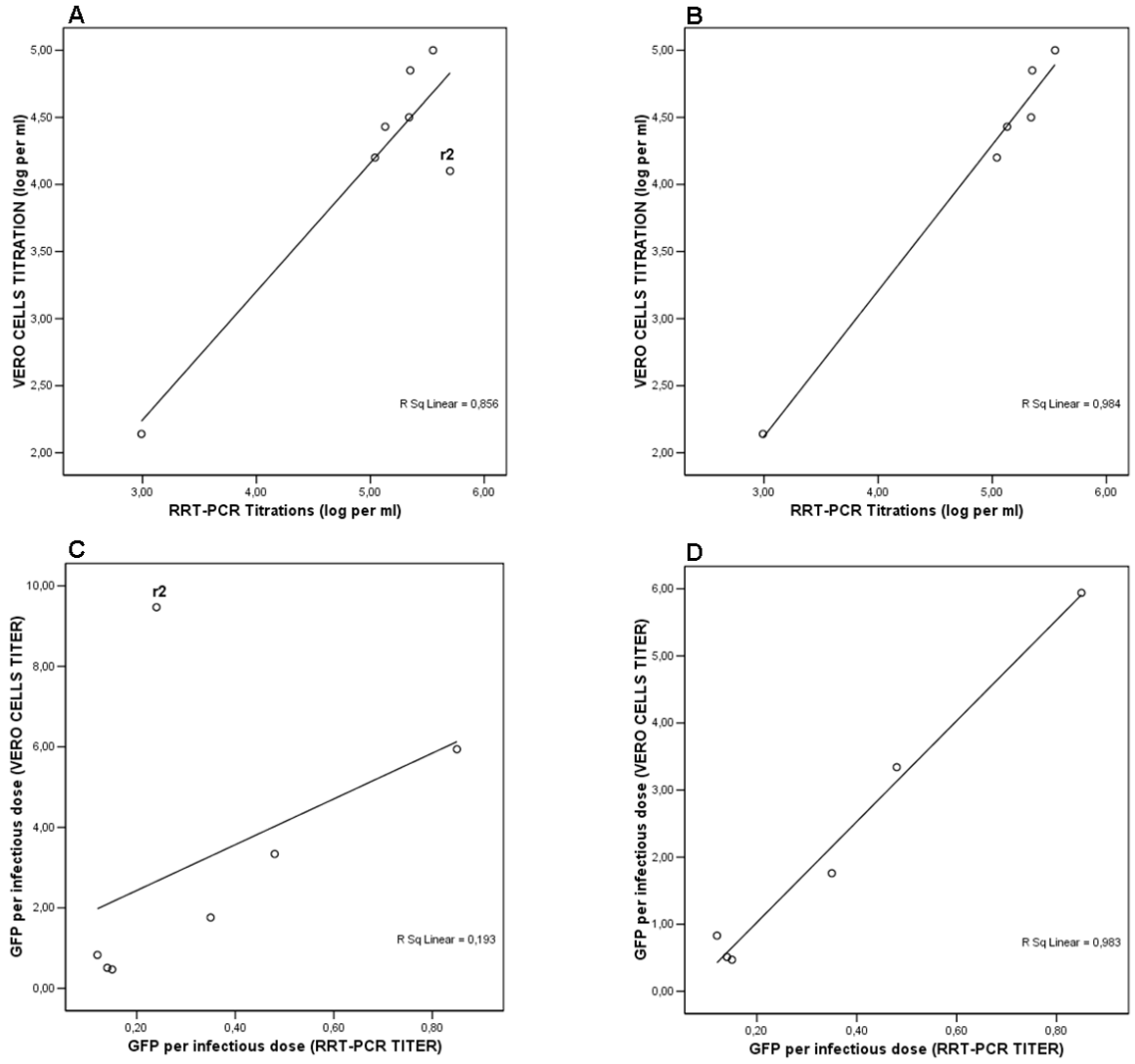

Figure 16: Comparison of GFP (pg) expressed by one infectious unit estimated by CPE in Vero cells with that estimated using real time RT-PCR. The Graphs show the correlation found between viral titers (A and B) and GFP expression (C and D), including (A and C) or not including (B and D) r2 values.

mRNA RT-PCR. All upstream gene stop signals were capable of stopping polymerase transcription as showed by the strong PCR bands visible on agarose gel (Figure 17).

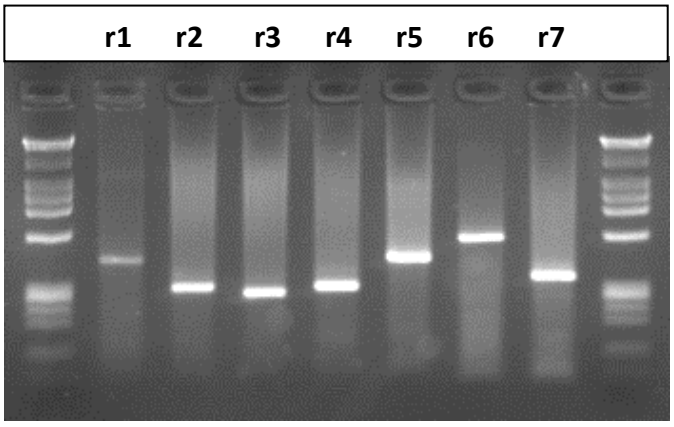

Figure 17: Specific upstream mRNA RT-PCRs bands viewed on agarose gel

3.5 DISCUSSION

Seven different AMPV cDNAs were modified, each one in a different IGR, in order to accept and express the GFP reporter gene. A cassette was firstly introduced adding a restriction enzyme site, for actual genic sequence insertion, and transcriptional start and stop signals, essentials for gene transcription. GFP was then inserted in all constructs and, after viral rescue, seven viruses carrying the reporter gene were obtained. Expression was assessed firstly by the presence of fluorescence under a UV microscope and later using a specific GFP

ELISA, able to quantify the amount of protein produced. Viruses were titrated using both CPE endpoints on Vero cells and real time RT-PCR, to calculate the amount of protein produced by a single infectious viral dose, and sequenced to detect possible occurrence of mutations during viral passages.

This study demonstrated the capacity of AMPV to accept and tolerate insertions in all the IGRs. The insertion process appeared to be very easy and both transcriptional signals present in the cassette proved to be effective for gene transcription, suggesting that the same system could be used for adding other genes, expressing, for example, foreign viral proteins. After rescue, CPE was seen in Vero cells infected with all recombinants and evidence of GFP was confirmed by observing strong fluorescence in all recombinants. Recombinant viruses were passaged three times and titrated in Vero cells and obtained titres were interpreted as an indicator of viral viability in vitro. All viruses, except r1, replicated in Vero cells to high titres (between 4 and 5 $\log_{10}$ per ml), with r3 producing the highest titre. The insertion of GFP between N and P significantly reduced the titre of r1 to less than 3 $\log_{10}$ per ml, which was statistically different from the others. These data are similar to those reported in the construction of VSV recombinants (232). N and P are in fact two essential components of the ribonuclear complex (RNP), which is critical for genome replication and transcription. Studies on VSV have shown the molar ratio between these two proteins to be critical for RNP functionality and an insertion in that position seems to significantly alter this ratio, causing reduced virus production (106, 174) and this might explain the low titre.

Although all viruses showed the capacity to carry and express GFP, quantification of this expression was performed by ELISA and results were

compared to the total amount of virus in order to assess the actual expressing efficiency per single infectious viral dose. AMPV is a NNS RNA virus and in such viruses, gene transcription and expression are regulated by gene position with respect to the polymerase single entry point at 3' end. In this type of virus 3' proximal genes appear to be the most transcribed while the distal 5' ones are the least, with a sequential and gradual decrease at every gene junction of 20 – 30 % in terms of transcription rate, due to polymerase dissociation (108, 233). Therefore, we expected to detect the best GFP expression efficiency in r1 and least in r7, with the other recombinants varying GFP expression according to the distance of the inserted gene from the 3' end.

For a correct efficiency estimate, it was essential to define the exact viral titre, as insertion of foreign sequences in certain positions can severely affect viral biology (232). Poor replication and viability would result in fewer viral particles being produced and, as consequence, in less total proteins expressed leading to an underestimation of this efficiency. On the other hand, an undercalculated virus amount would produce a greater total amount of proteins, causing an overestimation. As already explained above, viruses were titrated firstly on Vero cells and secondly, for an improved accuracy, real time RT-PCR was performed. One method relies on the viral capacity to infect Vero cell monolayers and the other detects the viral genome quantity by PCR. While the first titrates viruses by assessing the presence of infective virus with ten-fold dilutions and detects the end point of particles able to cause CPE, the latter detects the presence of viral RNAs, including those encapsidated in virions, which, for different reasons, do not cause CPE, but are at least potentially able to transcribe and express genes.

Real time RT-PCR gave higher titres with values between 5 and 6 $\log_{10}$ copies per ml, compared to the 4 and 5 $\log_{10}$ $TCID_{50}$ reached on Vero cells. This is easily explained considering the different scientific principles standing behind each method. However, statistical analysis showed a good correlation when comparing both titres of each recombinant, proving the accuracy of our estimate. Only r2 showed a large discrepancy between the two values. This virus had GFP inserted between P and M and it might be speculated that an insertion in that position could decrease the synthesis of M protein, which appears to be critical for viral assembly (171, 210). This would not affect the amount of RNA produced, as showed by the real time RT-PCR, but reduces in less infectious virions and lowers CPE endpoints.

GFP ELISA results were compared to both viral titres to calculate the expression efficiency per single infectious dose. As shown in Figures 14 and 15, trends were identical for both methods, with the only exception of r2, where, as explained above, virus titres in Vero cells was probably underestimated, gaving an overrated GFP expression per dose. The above speculation was suggested even by statistical analysis as seen in Figure 16. Excluding for this reason r2, r1 showed the highest level of expression efficiency, followed by r6 and r7, while r3, r4 and r5 appeared to have a very poor expression. Surprisingly, these findings do not match with the current transcriptional model for NNS RNA viruses. Although r1 was expected to produce the greatest amount of GFP per infectious dose and actually did, however it does not seem clear why r3, r4 and r5 showed such low levels. Furthermore this theory does not explain either why, r6 and r7 are respectively the second and the third viruses for expression efficiency. It might be speculated that AMPV does not respect the established

model, and perhaps the polymerase might entry the genome in an other position, for example prior to SH-G junction. This might clarify the r6 and r7 results, but would not totally explain r3, r4 and r5 GFP levels.

A different explanation, which complies with polymerase single entry theory, might elucidate the trend observed in Figures 14 and 15. Looking just at r1, r6 and r7 values, it is possible to recognize a decreasing gradient, where r1 is the highest point and r7 the lowest. r3, r4 and r5 low levels might be explained by the presence of a non-effective stop signal of the previous gene. In that case the majority of the polymerase molecules would read through GFP, transcribing a high amount of dicistronic messenger RNA. Viral mRNA including two genes leads at ribosomial stage to the synthesis only of the protein encoded by the first one, considerably reducing the amount of GFP. For this reason, the upstream gene mRNA RT-PCR was performed for all recombinants. Good bands were seen for all constructs proving the functionality of the stop signals. However as proven by the fluorescence observation, GFP expression has been seen with all viruses, meaning there could be a mixed population of GFP dicistronic and monocistronic mRNA. Different percentages of dicistronic and monocistronic GFP messenger might than explain the differences found in protein values per dose among recombinants. However to be able to prove that, other techniques such as Northern Blot or mRNA real time PCR should be employed.

Alternatively instability problems of the viruses, related to the insertion, might have explained this picture, but sequence analysis showed no mutations, proving the high stability of recombinants derived by NNS RNA viruses, as reported by previous studies (192).

In conclusion, seven different AMPV recombinants expressing GFP in different genome position were constructed and GFP expression efficiency was assessed by ELISA results compared to viral titres. The virus with the insertion between N and P (r1) proved to produce the highest quantity of reporter protein per infectious viral dose, followed by the one having GFP between SH and G (r6). However, as r1 showed a very low viral viability in vitro, r6 proved to be a better vector virus, resulting in SH and G to be the best likely position for insertion of extra genes. Future work is essential to confirm these findings in chickens, testing if the outcomes found *in vitro* have effects on animal models, especially in terms of immunological induction. This appears to be crucial for the future development of recombinant vaccines using AMPV.

CHAPTER 4

AVIAN METAPNEUMOVIRUSES EXPRESSING *INFECTIOUS BRONCHITIS VIRUS* GENES ARE STABLE AND INDUCE PARTIAL PROTECTION

TABLE OF CONTENT

4.1 ABSTRACT

This study investigates the ability of subtype A of *Avian metapneumovirus* (AMPV) to accept foreign genes and be used as a vector for delivery of genes from *Infectious bronchitis virus* (IBV) QX to chickens. Initially the green fluorescent protein (GFP) gene was added to AMPV at all gene junctions in conjunction with the development of cassetted full length DNA AMPV copies. After recombinant virus had been recovered by reverse genetics, GFP positions supporting gene expression while maintaining virus viability *in vitro*, were determined. Subsequently, either S1 or nucleocapsid (N) genes of IBV were positioned between AMPV M and F genes, while later a bivalent recombinant was prepared by inserting S1 and N at AMPV MF and GL junctions respectively. Immunofluorescent antibody staining showed that all recombinants expressed the inserted IBV genes *in vitro* and all recombinant viruses were found to be highly stable during serial passage. Eyedrop inoculation of chickens with some AMPV-IBV recombinants at one-day-old induced protection against virulent IBV QX challenge 3 weeks later, as assessed by greater motility of tracheal cilia from chickens receiving the recombinants. Nonetheless evidence of AMPV/IBV seroconversion, or the replication of recombinant viruses in trachea, were largely absent.

4.2 INTRODUCTION

Avian metapneumovirus (AMPV) is a major endemic respiratory pathogen of global domestic poultry with the exception of Australasia (14), with most severe disease occurring in turkeys, but also causing respiratory disease and reduced egg production in chickens. In both species, secondary infections can play an important role in exacerbating the disease (50). AMPV is an enveloped negative sense single stranded RNA virus belonging to the subfamily *Pneumovirinae*, genus *Metapneumovirus* (178) and four subtypes have been recognized (A, B, C and D) based on nucleotide sequence analysis (12, 122, 193). The genome comprises 8 genes with most subtypes having a genome size close to 13.5 kb (70).

The construction of reverse genetics (RG) systems for subtypes A and C has allowed those AMPV genomes to be rationally modified (92, 158). To date RG has produced viruses with deletions, gene modifications and reporter gene insertions (23, 92, 133, 163). Some studies have also given an indication of replicative ability of recombinants in vivo (133, 163) but there have been no reports concerning the genome's ability to accept extra viral genes, or the genetic stability of such recombinant viruses. In the current study, two genes from infectious bronchitis virus (IBV) were inserted into the AMPV genome. IBV is a major pathogen of chickens worldwide, which like AMPV, primarily infects the respiratory tract, while the former, also has the ability to infect the kidneys, intestine and reproductive system (35). Epidemiology is characterized by the frequent emergence of new

variants, of which some have major disease significance due to being able to avoid protection induced by prevailing vaccines, in combination with an ability to cause pathogenic effects. An example in the late 1980s/early 1990s was the emergence of the 793B/4-91/CR88 genotype (28, 59) which caused notable disease on farms in Europe with associated economic losses and led to the generation of new vaccines. More recently this trend has been manifested by the emergence of the QX genotype (238), and genes from this genotype were utilized in the current study.

The major viral surface protein is spike, which is coded by a single gene producing a precursor protein S0, which is cleaved into S1 and S2. The former is outermost on the virus and influential in IBV antigenicity (35). The internal nucleocapsid (N) protein can also induce protective immunity (199, 242) and that immunity has been shown to involve both T and B lymphocyte epitopes (199, 241). In the present study three AMPV genomes were modified to accept the IBV QX genes. Virus A was a German field isolate subsequently passaged in Vero cells (158), which was found to be avirulent in turkeys (163). Virus A vF was an F #8544 gene modification of A and found to dramatically increase induced immunity in turkeys while inducing a very minor increase in virulence (163). Virus 309/04 was a virulent field isolate derived from an unrelated subtype A vaccine (30). Hence for the study, three genome types from viruses with a spectrum of virulence were employed.

Prior to introduction of IBV QX S1 or N genes, a cloning site flanked by a transcription start and stop sequence was added to the intergenic regions of the full length (FL) DNA genome copies (cDNAs) of AMPV. Initially GFP was added to these sites and recombinant viruses were recovered, both as proof of principle

and to aid future vaccine optimization; then AMPV-S1-IBV and AMPV-N-IBV recombinant viruses were similarly constructed. Two versions of the S1 gene of IBV were inserted and used because sequencing of the selected QX genotype revealed that this gene comprises two sequence populations present, with one containing a 15 nucleotide deletion.

Recombinant viruses were used to inoculate chickens to determine their ability to induce protective immunity against IBV challenge in two different experiments. Inoculation of chickens with most IBV field strains leads to infection of and damage to the tracheal ciliated epithelium (65). This loss of cilia and/or motility is readily observed by low power microscopy (37) and induced protection is now considered to be most reliably confirmed by the maintenance of motility following virulent challenge (78) as was taken into account in European Pharmacopoeia, IBV vaccine monographs. To assess replication of AMPV-IBV recombinants prior to challenge, real time RT PCR was performed on material from choanal swabs and specific antibody responses to IBV and AMPV were measured by ELISA and hemaglutination inhibition (HI).

4.3 MATERIALS AND METHODS

Addition of GFP to AMPV. Using site directed mutagenesis, seven versions of a virus A (163) FL cDNA were made. A Xho1 restriction endonuclease (RE) site was added between each gene in the untranslated region, following the

leader proximal gene stop signal and prior to the downstream gene start signal. A cloning cassette flanked by cut Sal 1 RE sites, allowing foreign gene insertion and transciption, was ligated into each cut Xho1 RE site of the seven FLs. After cloning into STB12 cells (Invitrogen), the seven FLs were cut with Sal 1. GFP genes flanked by Xho1 sites were produced by high fidelity PCR using primers GFP ins + and GFP ins neg (Table 4) and these were added to the seven A varaint FL cDNAs by ligation. After cloning, colonies containing DNA of correct orientation and sequence were selected by PCR and sequencing. FLs containing GFP were then recovered by RG (158). Expression of the reporter gene was assessed by fluorescence microscopy. Virus viability was evaluated as maximum titers obtained in Vero cell monolayer titrations following three passages. The insertion process is outlined in Figure 18.

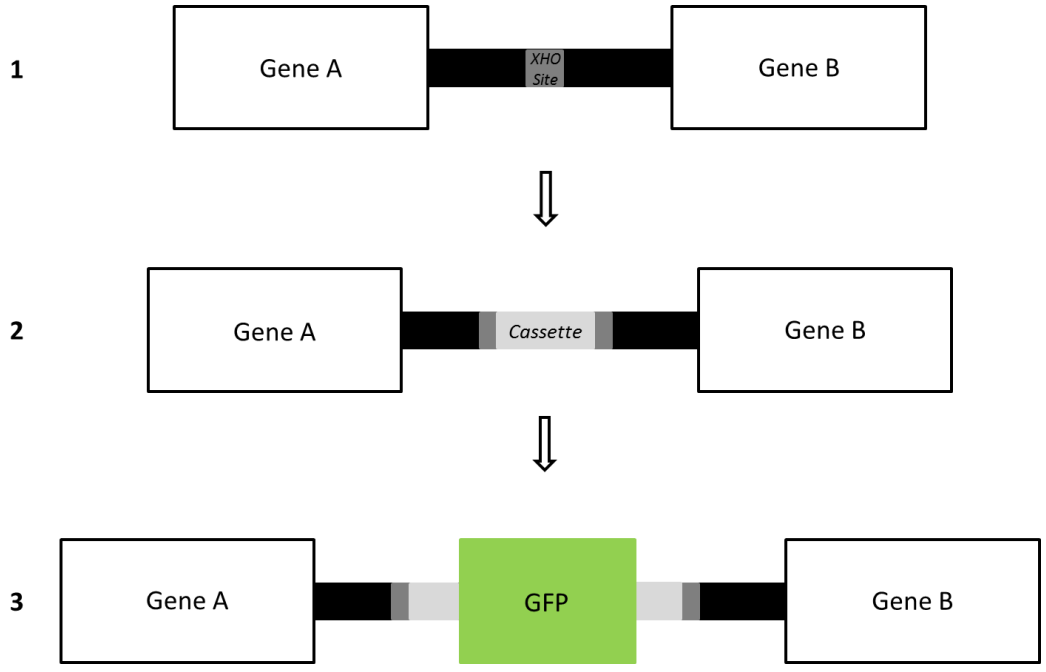

Figure 18: Schematic representation of the strategy used for adding genes to cloned AMPV genome

Table 4: Oligonucleotide sequences of primers used in this work

	Name	**Primers (5'… 3')**	**Function**
1	GFP ins +	GGGACCTCGAGTATGGTGAGCAAGGGCGAGGAGC	GFP amplification adding XhoI sites
2	GFP ins neg	CCACTCCTCGAGATTTTACTTGTACAGCTCGTCC	GFP amplification adding XhoI sites
3	N all b neg	ACTAATGAGAATCACAATAATAAAAAGCACAG	N RT and PCR amplification
4	N 200 +	GCAGCATGGATACTGGAGACG	N sequencing
5	N 300 neg	GGTCAGCGGCTGGTCCTGTTCC	N sequencing
6	N 560 +	GGTTCACGTGGTCGTAGGAG	N sequencing
7	N 750 +	CCAGGTTATAGAGTAGATCAAGTATTTGGC	N sequencing
8	N 920 +	CTGTGGTGCCTAGAGATGACC	N primer for mRNA PCR
9	N all +	CCAAGGGAAAACTTGTGAGGAACAC	N PCR amplification
10	N start xho +	GGAACACTATTATAATAACAATCCTCGAGCATGGCAAGCAGTAAGG	N amplification adding sticky ends
11	N stop xho neg	TGTAGCAAGTCCTTACTCGAGTCAAAGTTCATTTTCACCAAG	N amplification adding sticky ends
12	QX 1210 neg	ACATTCAAAATTCATGCTTAA	Diagnostic RT-PCR for QX IBV
13	QX 860 +	TGTTAATACTACTCTGGCG	Diagnostic RT-PCR for QX IBV
14	QX S1 1050 +	GGTTTAATTCCTTGTCAGTTTCTCTTACTTATGG	S1 sequencing
15	QX S1 1380 +	GCTGCTAATTTTAGTTATTTAGCAGATGGTGG	S1 sequencing
16	QX S1 270 neg	CCTGAAGAGGTGCTGTCATAGC	S1 sequencing
17	QX S1 400 +	GGCATGATTCCACGTGATCATATTCG	S1 sequencing
18	QX S1 550 neg	CAGTAGTTTTGTTGGAAGTAAAAACAAGATCACC	S1 sequencing
19	QX S1 end neg	CGAACCATCTGGTTCAATACAAAATCTGC	S1 PCR amplification
20	QX S1 start +	CCAGTTGTGAATTTGAAGAAAGAACAAAAGACCGACTTAG	S1 PCR amplification
21	RT QX S1 neg	CATCTTTAACGAACCATCTGG	S1 RT amplification
22	S1 1380 +	GCTGCTAATTTTAGTTATTTAGCAGATGGTGG	S1 primer for mRNA PCR
23	S1 start xho +	GGTAAATTATTGCTCGAGGATGTTGGTGAAGTCACTGTTTTTAGTG	S1 amplification adding XhoI sites
24	S1 stop xho neg	GTTACGTTTTGCTCGAGTTAACGCCTACGACGATGTGAGCTATTGG	S1 amplification adding XhoI sites
25	SX 3 +	TAATACTGGYAATTTTTCAGA	S1 sequencing

Analysis of IBV QX genes prior to insertion into AMPV. An IBV QX virus isolated in Germany was grown in eggs, titrated in tracheal organ cultures (TOC) and stored in aliquots at -80^{o}C. As the viral RNA sequence was unknown, a range of IBV genotype sequences were aligned to identify conserved regions within, and flanking, S1 and N genes. These were used to design RT-PCR and sequencing primers, as detailed in Table 4. Gene amplification and sequencing revealed the presence of two S1 populations, one of which had a deletion of 15 nucleotides. Table 5 summarizes the differences found.

Table 5: RNA and predicted amino acid differences between the two S1populations.

Position	Full S1		Deleted S1	
	Nucleotides	AA	Nucleotides	AA
8	U	V	G	G
58-75	U**UG UUU GAU UCU GAU A**AU	LFDSDN	UAU	Y
806	C	T	U	I
1181	U	M	G	R

Predictive computational comparison of S1 populations. The S1 gene containing the 15 nucleotide deletion was compared to the intact gene. The Protean program of the DNASTAR multiple program package (Lasergene Inc., USA) was used to estimate physicochemical properties, composition of the proteins and prediction of secondary structures. Order–disorder predictions were obtained using the VL-XT predictor on the PONDR server (185). Prediction of immunodominant helper T-lymphocyte antigenic sites from primary sequence data was carried out by analysing the occurrence of amphipathic fragments using the AMPHI algorithm (141). For all analyses, the predictive algorithms had been previously shown to be correct in more than 75% of cases.

S1 and N IBV amplification for insertion. For gene insertion, Xho I RE sites were added to S1 and N gene extremities during RT-PCR amplification by use of modifying primers S1 start xho +, S1 stop xho neg, N start xho + and N stop xho neg (Table 4).

IBV gene insertion into AMPV cDNAs. IBV genes were inserted into cloned FL A with the cassette at the MF intergenic region, as well as into identically modified FL A vF (163) or FL 309/04 (30). For insertion of a second gene, the cloning cassette was additionally added between G and L genes. After cloning into STB12 cells, seven recombinant cDNAs were produced as detailed in Table 6.

Table 6: Summary of the constructed recombinant cloned FL cDNAs

Clone name	Source virus	IBV QX insert	Intergenic region
A $_{\text{del S1 MF}}$	A	Deleted S1	MF
A $_{\text{full S1 MF}}$	A	Full S1	MF
A vF $_{\text{del S1 MF}}$	A vF	Deleted S1	MF
A vF $_{\text{full S1 MF}}$	A vF	Full S1	MF
A vF $_{\text{N MF}}$	A vF	N	MF
A vF $_{\text{full S1 MF + N GL}}$	A vF	Full S1+ N	MF + GL
309 $_{\text{full S1 MF}}$	309/04	Full S1	MF

Recovery of virus. IBV recombinant viruses were rescued using the modified plasmids as previously described and subsequently passaged in Vero cells to produce sufficient virus for protection studies. Viruses were titrated in 48 well plates containing Vero cell monolayers; cytopathic effect end points were

observed using low power microscopy and titers were calculated (183). Viruses were aliquoted in 1.5 ml screw top tubes and frozen at -80^{o}C.

S1 and N gene messenger RNA RT-PCRs. Specific mRNA RT PCRs (23) were used to verify the transcription of inserted genes. Messenger RNA was amplified using a previously reported protocol (23) except that primers within reading frames were substituted by S1 1380+ and N 920+ for S1 and N genes respectively (Table 4).

Expression of inserted genes. S1 and N protein expression were assessed using immunofluorescence (IF) techniques on AMPV recombinant infected Vero cells. A polyclonal chicken antiserum (GD) anti QX was used to evaluate S1 expression while this was substituted by a monoclonal mouse antibody (Biozol) for the N gene. Suitable FITC conjugated anti chicken/mouse antibodies were used according to manufacturers' protocols to enable visualization of specific S1/N proteins.

Chickens experiment 1. Approximately 70 one-day-old SPF chickens were assigned to seven groups of ten. In groups one to four, birds were inoculated by eyedrop with 4 $\log_{10}$ $TCID_{50}$ A $_{full\ S1\ MF}$, A vF $_{full\ S1\ MF}$, A $_{del\ S1\ MF}$ and A vF $_{del\ S1\ MF}$ respectively. The AMPV control group was inoculated with A vF. The remaining positive and negative control groups were inoculated with sterile water. At 21 days post vaccination (dpv), all birds, except the negative controls, were challenged with 4 $\log_{10}$ $TCID_{50}$ QX IBV strain. For sampling, half the chickens in each group were humanely culled at 4 days post challenge (dpc) and the remainder at 6 dpc.

Chickens experiment 2. Approximately 60 one-day-old SPF chickens were assigned to six groups of ten birds, four of which were inoculated with 4 $\log_{10}$

$TCID_{50}$ of viruses 309 $_{full\ S1\ MF}$, A vF $_{full\ S1\ MF\ +\ N\ GL}$, A vF $_{N\ MF}$ and A vF $_{full\ S1\ MF}$ virus by eyedrop. The remaining groups acted as controls and were inoculated with sterile water. At 21 dpv, all the birds, except the negative controls, were challenged with 4 log_{10} $TCID_{50}$ of IBV QX. In each group, birds were humanely killed five at 4 dpc and five at 6 dpc.

Clinical signs. Birds were observed daily for clinical signs both after vaccination and after IBV challenge.

Serology. Chickens from each group were bled at 18 dpv for IBV and AMPV serology. Sera from birds which received S1 recombinant AMPVs were tested using an IBV QX HI test, while sera from birds receiving either recombinants containing the N gene were tested for anti IBV antibodies by HI and ELISA (Biochek). AMPV ELISA (IDEXX) was performed on all sera.

Assessment of replication of recombinants by AMPV real time RT-PCR. Ten choanal swabs were collected from all groups at 3, 6 and 9 days after vaccination for specific subtype A AMPV real time RT-PCR (40) to assess recombinant replication.

Determination of tracheal cilial activity. At 4 and 6 dpc in both chicken experiments, tracheas were collected and cut into 1 mm transverse sections. For each trachea, ten sections were collected (three upper, four middle and three lower) and examined under low power microscopy to determine activity of cilia. Each section was designated as either containing beating cilia or beating being entirely absent.

Statistical analysis. Statistical analyses were performed for ciliar motility results using the Chi-square test. A p value < 0.05 was considered statistically significant.

4.4 RESULTS

Recombinant AMPV GFP construction. GFP was added to modified FL AMPV copies and viruses were recovered in the reverse genetic system. Maximum $TCID_{50}$ titres per ml of Vero cell lysate achievable following three Vero cells passages were generally lower near to the virus leader, then increased; hence with GFP at the NP position the titre was 2.1 log $TCID_{50}$ while in all other positions it exceeded 4.0 log $TCID_{50}$ with the highest being for the MF position which gave a titre of 5.0 log $TCID_{50}$. Strong fluorescence was observed when infected Vero cell monolayers were viewed by UV microscopy for all constructs. Figure 19 shows the fluorescence for the GFP MF recombinant.

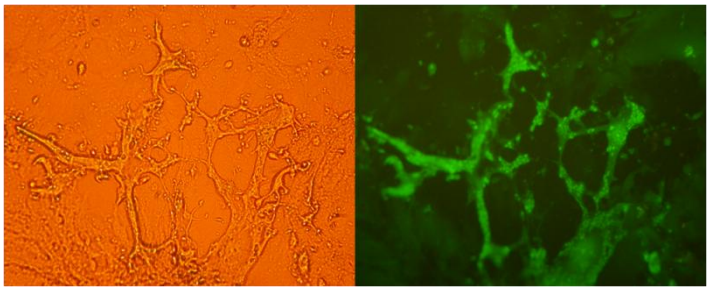

Figure 19: Vero cell monolayer infected with virus expressing GFP between MF viewed under white (left) and ultraviolet illumination (right).

Computational analysis. Protean analysis of S1 proteins showed both clones shared the same physicochemical properties and similarly PONDR predicted no differences to the disordered region. Furthermore the Protean suite predicted the proportion of alpha helix to remain constant but not for beta pleated sheets where a difference was evident at the beginning of the two proteins. The AMPHI program predicted a T cell epitope present only in the complete S1 protein, at amino acid positions 15-19. However S1 containing the deletion possessed two additional predicted T cell epitope regions (amino acid positions 264-268 and 383-388).

IBV recombinant AMPV construction. AMPV FL cDNAs containing IBV genes were constructed which led to virus recovery as confirmed by cytopathic effect typical of AMPV on Vero cell monolayers. Viruses were sequenced in order to exclude the occurrence of mutations during the three passages. RT-PCR of virus mRNAs confirmed transcription of the inserted IBV genes (Figure 20). Expression of IBV proteins was confirmed in all recombinant viruses by IF as shown in Figure 21.

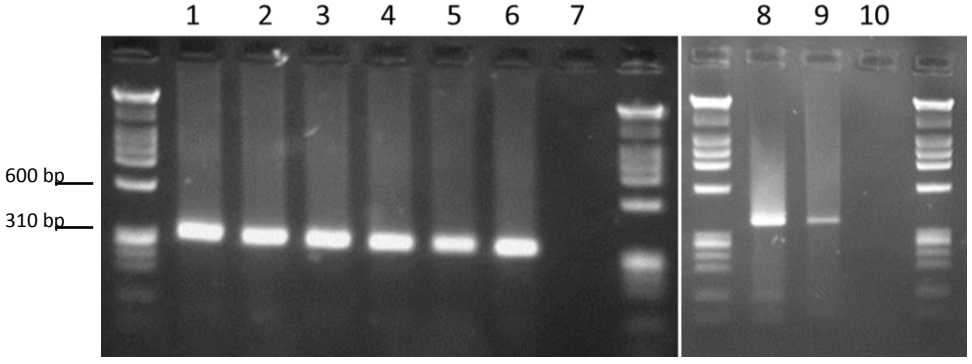

Figure 20: PCR products of 7 AMPV-IBV recombinants using primers for the S1 and N genes of IBV. Specific messenger RNA RT-PCRs for S1 are showed in lanes from 1 to 7 while N gene ones in lanes from 8 to 10. Viruses tested : 1 - 309 $_{full\ S1\ MF}$*, 2 - A vF* $_{full\ S1\ MF}$*, 3 - A vF* $_{full\ S1\ MF\ +\ N\ GL}$*, 4 - A vF* $_{del\ S1\ MF}$*, 5 - A* $_{full\ S1\ MF}$*, 6 - A* $_{del\ S1\ MF}$*, 7 - S1 negative AMPV control, 8 - A vF* $_{N\ MF}$ *and 9 - A vF* $_{full\ S1\ MF\ +\ N\ GL}$*. Lane 10 - negative AMPV control.*

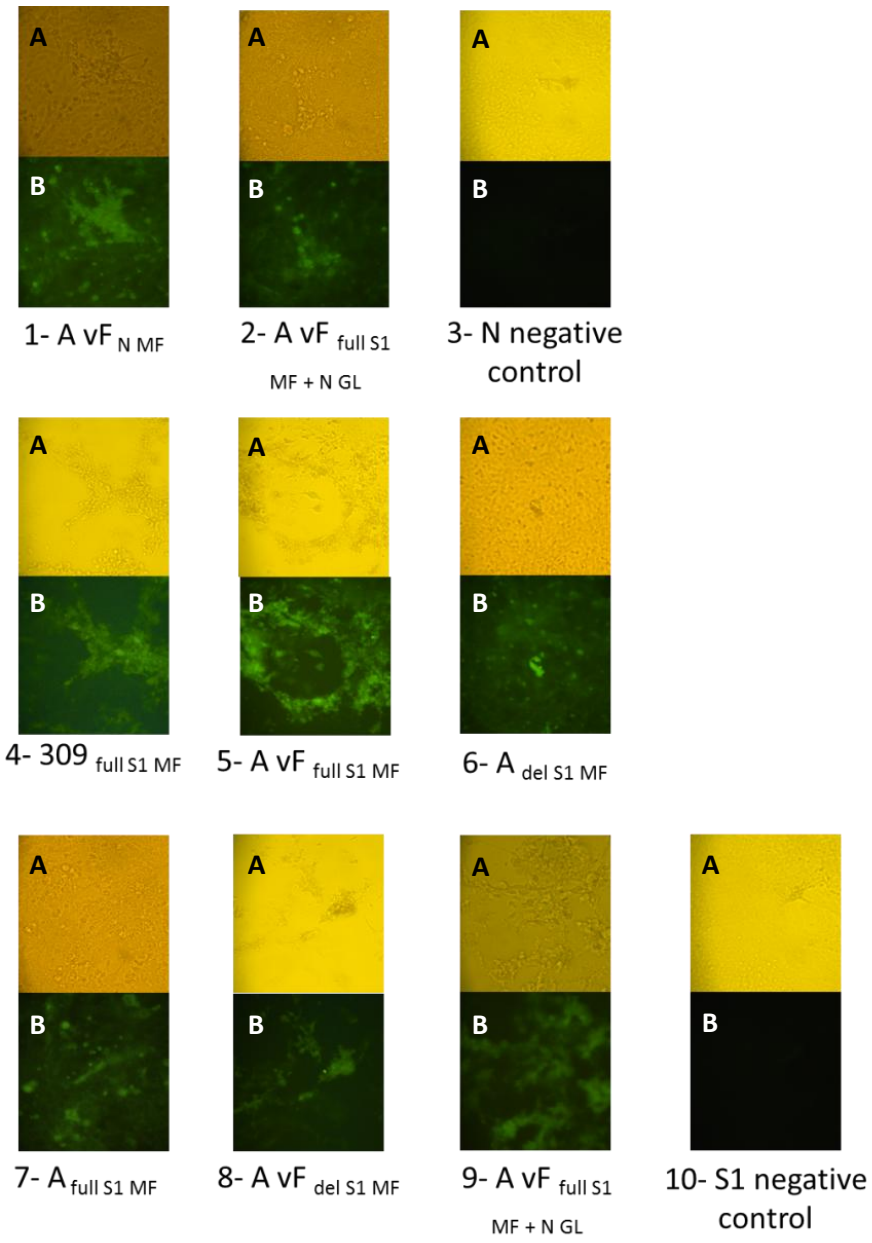

Figure 21: Microscopy of VERO cells infected with 7 AMPV-IBV recombinants and negative controls. Top A: white light illumination; B: Immunofluorescence using FITCH conjugated specific antibodies. Viruses 1 to 3 used IBV N monoclonal serum and 4 to 10 used IBV polyclonal chicken serum

Serology. In general IBV and AMPV antibody responses were not detected in either chickens experiments, either by HI or ELISA (Table 7). For IBV in Experiment 1, a single bird in group A vF $_{\text{del S1 MF}}$ and two birds in group A vF $_{\text{full S1 MF}}$ had detectably seroconverted by HI. For AMPV ELISA serology, two seroconversions were detected in the A vF control group (Experiment 1) and three in the 309 $_{\text{full S1 MF}}$ group (Experiment 2).

AMPV real time RT-PCR. Real time RT-PCR showed minimal replication for all AMPV recombinants with the exception of 309 $_{\text{full S1 MF}}$, which was replicated in most birds (Table 7).

Tracheal motility following challenge. The percentages of rings showing cilial activity are given in Table 7. At day 4, sections from all birds challenged with IBV were ciliostatic. At day 6, some sections from birds previously inoculated with AMPV QX recombinants showed recovered cilial motility. Stronger tracheal recovery was observed in those birds given A vF $_{\text{full S1 MF + N GL}}$, followed by A vF $_{\text{N MF}}$ with the least seen in A vF viruses, which express only the S1 gene. There were no noticeable differences between recombinant AMPVs expressing the full and the deleted S1. When comparing AMPV vectors with identically positioned inserts, recombinant vector A led to greater cilial recovery than recombinant of either 309/04 vector or vector A vF.

Statistical analysis. Significant p values, calculated comparing each group to the respective positive control, were found only for birds vaccinated with A $_{\text{full S1 MF}}$ ($p = 0.0002$), A $_{\text{del S1 MF}}$ ($p = 0.0008$), A vF $_{\text{full S1 + N GL}}$ ($p = 0.00001$) and A vF $_{\text{N MF}}$ ($p = 0.0002$). Analysis between these four groups did not reveal any significant differences.

Table 7: Effects of vaccination with 7 AMPV-IBV recombinants on virus replication, antibody response to IBV and AMPV and on % TOC beating after challenge with IBV.

	Groups	**AMPV real time RT PCR** (dpv[1])			**Serology** (18 dpv[1])			**% TOC beating** (dpc[2])	
					AMPV	**IBV**			
Experiment 1		Day 3	Day 6	Day 9	ELISA	HI	ELISA	Day 4	Day 6
	A $_{\text{del S1 MF}}$	4/10	0/10	0/10	0/10	0/10	n.d.*	0	20
	A $_{\text{full S1 MF}}$	0/10	0/10	0/10	0/10	0/10	n.d.*	0	24
	A vF $_{\text{del S1 MF}}$	0/10	0/10	1/10	0/10	1/10	n.d.*	0	0
	A vF $_{\text{full S1 MF}}$	0/10	0/10	0/10	0/10	2/10	n.d.*	0	0
	A vF	0/10	0/10	5/10	2/10	0/10	n.d.*	0	0
	C +	0/10	0/10	0/10	0/10	0/10	n.d.*	0	0
	C -	0/10	0/10	0/10	0/10	0/10	n.d.*	100	98
Experiment 2	A vF $_{\text{N MF}}$	0/10	0/10	0/10	0/10	0/10	0/10	0	32
	A vF $_{\text{full S1 MF + N GL}}$	0/10	0/10	0/10	0/10	0/10	0/10	0	40
	309 $_{\text{full S1 MF}}$	6/10	8/10	6/10	3/10	0/10	0/10	0	8
	A vF $_{\text{full S1 MF}}$	0/10	1/10	1/10	0/10	0/10	0/10	0	2
	C +	0/10	0/10	0/10	0/10	0/10	0/10	0	4
	C -	0/10	0/10	0/10	0/10	0/10	0/10	100	100

[1] days post vaccination

[2] days post challenge

* not done

C+ and C-: respectively non vaccinated challenged animals and non vaccinated non challenged birds

Clinical signs and gross lesions. No symptoms were detected after vaccination in both experiments. In Experiment 1**,** clinical signs were observed in three birds at 6 dpc. One bird in the positive control group and two birds in the A vF control group displayed severe depression. Birds showed lethargy, ruffled feathers and were not responding to major external stimuli and were humanly culled. Gross kidney lesions, typical of QX IBV infection, were detected at post-mortem examination in the same birds (Figure 22). No symptoms were seen in Experiment 2.

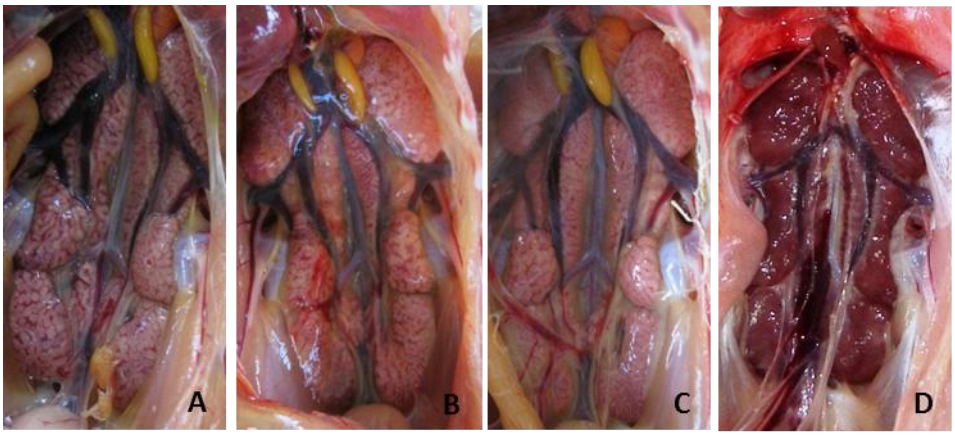

Figure 14: Comparison of gross lesions in kidneys detected in 1 bird from positive control (A) and 2 birds from AMPV control (B and C) groups, compared to a normal kidney from the negative control group (D), after challenge with QX IBV.

4.5 DISCUSSION

Stable recombinant AMPV viruses carrying GFP and IBV genes were generated, which were also demonstrated to express those inserted genes. IBV genes were inserted between the M and F genes of AMPV because insertion of the GFP gene in that position gave the most viable recombinant virus. When such IBV recombinants were used to inoculate one-day-old chickens, they induced IBV protection in the trachea. However serology and virus detection by real time RT PCR prior to challenge indicated that recombinant virus replication was generally very poor, and surprisingly, those replicating least well in the upper respiratory tract of chickens, induced most protection: hence IBV recombinants with AMPV virus A protected better compared to AMPV 309/04 IBV recombinant which induced no detectable protection. Despite computer analysis predicting differences of T cell epitopes between S1 populations with respect to the 15 nucleotide deletion, no differences were observed in terms of induced protection.

The primary site of both AMPV and IBV replication is the upper respiratory tract, hence an AMPV-IBV recombinant might be considered ideal for inducing IBV protection in chickens. For effective protection against IBV, the recombinant would be expected to be able to grow in those tissues and it appears possible that the very low level of replication observed in this study led directly to the less than optimal protection induced. An AMPV strain replicating better in chickens might yield more favourable results. Growing field evidence worldwide, gained by commercial farmers and vaccine companies, is indicating that subtype B viruses are more able to infect commercial chickens than the subtype A viruses used in the current study. However to date, there have been no reports of studies testing the relative ability of subtype A and B viruses to

replicate in commercial birds in experimental conditions. Once a subtype B RG system becomes available, it will be possible to test this assertion in parallel to the testing of subtype B virus recombinants. However, caution should be exercised in predicting likely outcomes because the A based recombinant AMPVs in the current study induced the best protection while not significantly replicating in the upper respiratory tract. In contrast, the AMPV recombinant based on isolate 309/04 replicated better but failed to induce detectable protection. If further recombinants are found which also induce protection without significant respiratory tract replication, it will become necessary to investigate whether virus replication occurs at another site or alternatively whether protection is induced by a so far unrecognized mechanisms. There is already some evidence that AMPV replication in the respiratory tract does not imply induction of AMPV protection (163) as well as the finding that AMPV protection following live vaccination does not require initial replication in the trachea (159). Furthermore AMPV vaccination can lead to apparent protection without seroconversion (62, 236).

For the first time, AMPV recombinants are reported carrying foreign viral genes. Previously AMPVs have been shown to be highly stable both in cell culture and during natural passage (30) as has also been reported for other members of the *Mononegavirales* such as vesicular stomatitis and rabies viruses (146, 191). This contrasts with single strand positive sense viruses such as IBV and feline calicivirus where even a few passages readily results in consensus sequence mutations (35, 180). In the current study it was further shown that a recombinant AMPV genome containing IBV S1 and N genes, totaling approximately 3000 extra nucleotides, was entirely stable with respect to those inserted genes, even though their sequences would be expected to be irrelevant to virus function and hence their random mutation would not be

expected to adversely affect virus viability. Furthermore the presence of those inserted genes did not appreciably reduce virus viability, so long as they were distanced from the leader. It is likely that placement closer to the leader reduced transcription rates of genes required in large quantities to enable optimal virus replication, such as the nucleocapsid and phosphoprotein genes (232).

The unproblematic construction of AMPV recombinant viruses suggests that it would be possible to generate a range of similar viruses with a view to either further RG studies or in order to construct a range of candidate vaccines for protection studies. Regarding the latter, the speed of construction is in marked contrast to conventional live IBV vaccines where more than 100 egg passages of field strains are generally required for suitable attenuation. In principle, a range of AMPV-IBV recombinant viruses could be readily made to determine all genes important in effective IBV protection. In contrast to the genetic instability characteristic of conventional live IBV vaccines, the use of AMPV recombinants allows the construction of vaccines of genetic stability carrying at least two IBV genes, and possible more. Furthermore the nature of AMPV replication implies that field recombinants, very often found during concurrent IBV infection and IBV vaccine use, would be highly unlikely to arise.

The nature of the protective immune response to IBV is not well understood (35). In the present study fast tracheal recovery was equally induced by the N gene or N + S1 genes inserted into virus A vF. However both induced markedly better protection than S1 in the same construct, while S1 placed in virus A gave a similar level of protection to N in A vF. This might imply that N or N plus S1 if inserted into virus A might induce much better protection than seen in our study. Previous studies indicate that the internal N protein is able to stimulate a cellular immune response (199) whereas the exposed S1

protein would be more likely to stimulate antibody production (36, 151, 152). However the S1 constructs inducing most AMPV and IBV antibody was based on 309/04 and A vF and these were the constructs which induced no detectable protection. Further studies will be needed before firm conclusions can be drawn in this area.

Because some of the subtype A viruses used to make recombinants are known to induce effective protection against AMPV in turkeys (163), it would be of interest to test the protection effects of subtype A recombinant viruses in that species. While IBV has no relevance to turkey disease, and AMPV recombinants carrying genes from coronavirus of turkeys could be substituted, agents causing disease associated with well-established challenge models such as *Newcastle disease virus,* would appear better choices.

In conclusion, we report for the first time that AMPV is able to accept genes from, express proteins of, and confer protection against foreign viruses. Furthermore the recombinant AMPV-IBV viruses are genetically stable despite the incorporation of additionally approximately 3000 nucleotides; the upper limit is yet to be established. As the recombinants tested were able to induce IBV partial protection, it is likely that further studies will lead to the production of flexible vaccines of greater efficacy as well as a better understanding of the essential components for induction of effective protection against IBV and other avian viruses.

CHAPTER 5

ATTEMPT TO DEVELOP A REVERSE GENETIC SYSTEM FOR *AVIAN METAPNEUMOVIRUS* SUBTYPE B

TABLE OF CONTENT

5.1 ABSTRACT

This work describes an attempt to develop a reverse genetic system for *Avian metapneumovirus* subtype B. The construction of a full length DNA copy (cDNA) of the viral RNA genome was attempted by firstly trying to exchange a previously developed subtype A genome cDNA by site directed-mutagenesis (SDM) in B subtype sequences. In a second stage, cut and ligation protocols relying on restriction endonuclease properties were employed. The development of DNA gene copies of the four essential support AMPV proteins (N, P, M2 and L) was also attempted using similar approaches. In order to stabilize and multiply SDM and ligation products, obtained constructs were cloned in commercial Stb12 *E.coli* after every reaction, with the intention of creating very stable bacterial plasmids. As a result of these processes, more than 85 % of B type genome was successfully cloned in the full length cDNA and three of the four support genes were obtained, namely N, P and M2. However, serious problems were faced with both the full genome and the L cDNA. Especially in the very late stages, cloning processes experienced unacceptable low success rate, suggesting a possible intolerance of viral sequences by the commercial bacteria.

5.2 INTRODUCTION

Recovery of an RNA virus from a DNA copy (cDNA) of its genome allows the direct determination of the effect and the function of artificially made genomic mutations on viral biological mechanisms. In contrast to RNA, DNA molecules are stable and easy to modify using several techniques such as site-directed mutagenesis or ligation protocols. In reverse genetics (RG), full viral genome cDNA can be copied to RNA using different strategies commonly relying on bacteriophage RNA polymerases (47). While for positive stranded viruses it has been easier to develop such systems since the RNA genome itself has the capacity to start an infectious cycle in cells, for negative stranded viruses the picture is more complex (229). Their genomes need to be associated with a ribonuclear protein complex (RNP) in order to initiate transcription and replication processes. RNP is usually formed by the nucleoprotein (N), which tightly encapsidates the viral genome, the polymerase (L) and the phosphoprotein (P), which acts as a polymerase-associated cofactor. Other proteins might be essential depending on the specific viral species (47, 48). Therefore RG systems for these viruses must not only provide the full cDNA genome as for positive stranded ones, but it is critical that all the proteins constituting the RNP are available.

Avian metapneumovirus (AMPV) is a non-segmented negative stranded virus, belonging to the *Paramyxoviridae* family, causing respiratory infections affecting turkeys and chickens (49, 155). In 2004 Naylor et al. developed the first reverse genetic system for AMPV (158). A full length (FL) cDNA of a subtype A viral genome was cloned in a plasmid by a series of PCR and ligation

steps. The plasmid included a kanamicyn-resistant gene, essential in the cloning process, a T7 promoter and *Hepatitis delta virus ribozyme* (158). Furthermore, N, P, L and Matrix 2 (M2) single genes, lead by a T7 promoter, were cloned in other plasmids in order to provide the genome with the essential protein to form the RNP complex. Viral rescue was performed on Vero cells previously infected with a recombinant *Fowlpox virus* expressing the bacteriophage T7 polymerase. This polymerase is able to recognize the T7 promoter inserted in the plasmids and then to initiate transcription directly from them. Therefore the addition of the full length cDNA and the four support protein genes, in the presence of lipofectamine 2000 to allow cDNAs entrance into cells, should lead to the formation of all the RNP components (158). After the complex has been established, genome replication and gene transcription can begin as occur naturally, producing new RNA virions. A similar RG system, based on the same technical principles, was developped for subtype C in 2006 in the United States (92). However, up to now it has not yet been possible to apply RG to subtype B. This subtype is one of the most widespread subtypes worldwide and, together with subtype A, is responsible for major production losses in poultry industries (50, 51). Two attempts to develop an RG system for this subtype, are believed to have been made, one by an Israeli research institute and another by an international veterinary vaccine company, but both are thought to have been terminated without final success (C.J. Naylor, personal communication).

This present study describes an experiment designed to develop a RG system for subtype B. The first step was the generation of full length cDNA subtype B and of the four support cDNA genes to be inserted into plasmids. Cloning whole viral genome sequences appeared to be very challenging in previous experiences, as for the construction of the full length subtype A cDNA (C.J. Naylor, personal communication) but even for other viral species, due to

the instability of these constructs resulting in mutations or deletions. One possible explanation is that the cloning process might not tolerate certain viral sequences and that these can encode for bacterial toxic peptides, resulting in cloning failure (48, 129, 149, 153). To overcome problems, several strategies have been applied; these include the use of low copy-number plasmids and very tolerant *E.coli* strains cultured at low temperatures (188). Moreover in view of previously gained experience with subtype A cDNAs construction, it was decided firstly to try to exchange the existing subtype A genome cDNA in subtype B using site directed mutagenesis techniques (SDM). This was attempted in several stages, with each one adding a further genome section. Success at each step was confirmed with specific subtype B PCRs and specific enzyme cuts in the modified plasmid. Then, in a second stage, protocols involving ligations of amplified PCR product were used.

5.3 MATERIALS AND METHODS

Subtype B viral strains. The vaccine strains Aviffa (Merial), Nemovac (Merial) and Rhino CV (Intervet) were used as sources of viral PCR products to be used for SDM and ligation reactions. RNA was extracted from these strains using a commercial kit (Qiagen).

Designed primers. Several oligonucleotide sequences were designed and synthesized by EUROFINS MWG OPERON company (Table 8). Primers that match both A and B subtypes were used to amplify amplicons for SDM reactions. For cut and ligations steps, primers adding at both ends restriction

Table 8: Oligonucleotide sequences of primers used for this study

OLIGONUCLEOTIDE SEQUENCES USED IN THIS WORK		
Name	Sequence (3'… 5')	Function
A 13.47 SAL +	caactgaccttaaatgaatgttagtcgacaaaaactaaaagc	Introducing RE site primer
A 8.51 SAL neg	cagcatccatggtcgacctttcttctac	Introducing RE site primer
B 0.03 +	acaagtcacaatagaaaagaga	Specific B type primer
B 1.0 SAL neg	gtgatctagagtcgacgcatgcaatc	Introducing RE site primer
B 1.43 +	ctatgtagctcagaaacttctag	Specific B type primer
B 10,1 neg	gtctgactcactgcctacttg	Specific B type primer
B 10,77 +	caattatcaaagcgcatacgggaaacat	Specific B type primer
B 11,1 neg	catgcagactagatctaaaagtctc	Specific B type primer
B 12 XHO +	ccatgtctgagtaactcgagcattatcacctcgaagattagtcccagctgcc	Introducing RE site primer
B 12.39 +	cagcataaaaagctgcctc	Specific B type primer
B 12.72 neg	gattaataaaagtacattatcagggtc	Specific B type primer
B 13,49-rib neg	gccgacccacggcaaaaaaaccgtattc	Both A and B type matching primer
B 13.49-rib+	gaatacggttttttgccgtgggtcggc	Both A and B type matching primer
B 2.04 neg	gtatctggttcagtctcttcac	Specific B type primer
B 2.2 +	cgatctagttgagaaggacaac	Specific B type primer
B 2.28 +	ctgctggaccagctaaaaactc	Specific B type primer
B 2.45 neg	ctgctcttacatcgcaaactgtgagtac	Specific B type primer
B 2.52 neg	ggtgttcatgtttgttacaatc	Specific B type primer
B 2.87 +	ccagagaactaggtatgtcc	Specific B type primer
B 2.97 +	caaactgctactaataatttatttggtgg	Specific B type primer
B 3.03 +	cttacagtgaagaatcatgcagcac	Specific B type primer
B 3.23 neg	cctatgggaaaggattcgattc	Specific B type primer
B 4.48 +	gggcatagcttactatgtggttaaaaag	Specific B type primer
B 4.84 neg	cccttctatataattctggacaacattggcag	Both A and B type matching primer
B 4.84 +	ctgccaatgttgtccagaattatatagaaggg	Both A and B type matching primer
B 4.94 neg	atctaccatcaggtctcgtgc	Specific B type primer
B 4.99 +	tgcataaccttgtcttgtcctatatagacatgag	Specific B type primer
B 5.48 neg	tcatagtccgaagacacagcatg	Specific B type primer
B 5.63 +	cctgagatcaacaagtcagcc	Specific B type primer
B 5.97 neg	ctaatctacttgtatgtatatactcatatg	Specific B type primer
B 7,36+	gaaagaagaagaacagcacacaacag	Specific B type primer
B 7,90 neg	attccaacagcttttacggagg	Specific B type primer
B 8,99 neg	taacactacatacttcttcaagttctcc	Specific B type primer
B 8.35 SAL +	gaacacatctgtcgaccatcatagatac	Introducing RE site primer
B 8.44 +	gctgcagagatgtattttatctttagaatattcggac	Both A and B type matching primer
B 8.44 neg	gtccgaatattctaaagataaaatacatctctgcagc	Both A and B type matching primer
B 8.53 +	cagtgaggccactaaaatattgagcctc	Specific B type primer
B 8.83 +	gtatatccaaaaaattatttacctccttctg	Specific B type primer
B 8.9 XHO +	gcagaacatgaaaaaactcgaggtgtactagagttttacttg	Introducing RE site primer
B 9,47 +	caaagggatttatgatattgactcaatccct	Specific B type primer
B 9,53 +	ggaccatggaggcaatatctctccttgatg	Both A and B type matching primer
B 9,53 neg	catcaaggagagatattgcctccatggtcc	Both A and B type matching primer

B 9.21 neg	cactcttcctagctttcac	Specific B type primer
B 9.59 +	ggttaagaacagggttcagcaa	Specific B type primer
B 9.7 +	cagactacagtttagcaataaaaatg	Specific B type primer
G 6.40 +	cgcagttatgctgtactgggtggtgttga	Both A and B type matching primer
G 6.40 neg	tcaacaccacccagtacagcataactgcg	Both A and B type matching primer
G 9+B	tagtcctcaagcaagtcctc	Specific B type primer
L 11.31 +	ggacattaacttggtcttccaaaatgctataag	Both A and B type matching primer
L 11.31 neg	cttatagcattttggaagaccaagttaatgtcc	Both A and B type matching primer
L end B neg	ctttatggtctattttgtgctcagtatgtacc	L gene amplification
L start B +	gaccaatatggacccatccaatgag	L gene amplification
M 2.12 +	gggacaagtaaacatggagtcct	Both A and B type matching primer + P gene amplification
M 2.12 neg	aggactccatgtttacttgtccc	Both A and B type matching primer
M 2.83 +	gagagcttagggaaaatatgcaaaacatgg	Both A and B type matching primer
M 2.83 neg	ccatgttttgcatattttccctaagctctc	Both A and B type matching primer
M2 start B +	gacaagtaaagatgtccagaaggaatccctg	M2 gene amplification
M2-1 end B neg	ttgcacctaattactgctgtcaccc	M2 gene amplification
N 8 +	gtgaaaatgtctcttgaaagtattaggctcagtg	Both A and B type matching primer + N gene amplification
NP 1.25 neg	gacattttcacttgtcccgaatttttaattactc	N gene amplification
P start B +	gtgaaaatgtctttccccgaaggcaag	P gene amplification
T7-21 +	ctataggacgagaaaaaaagcattcaag	Both A and B type matching primer
T7-21 neg	cttgtatgcgttttttttctcgtcctatag	Both A and B type matching primer

endonuclease (RE) sites were constructed. B type specific oligonucleotide sequences, which did not match with A type sequences, were designed to verify the presence of the modified sequences. Finally, amplification of the essential RNP component genes was carried out using B subtype-specific primers for N, P, M2 and L.

RT-PCRs. RT- PCR protocols were assessed to amplify variable size B type amplicons. In order to improve PCR efficiency, annealing temperatures were adjusted to primer characteristics and elongation steps were optimized according to the expected amplicon size. Different polymerases were employed, e.g. GoTaq Flexi DNA (Promega), Bio-X-act (Bioline) and PFU turbo (Stratagene) due to the different properties of each enzyme: e.g. the last named enzyme was used preferentially as it performs high fidelity long

amplification and, contrary to other polymerases, it does not add overhanging ends to the final product.

Subtype A FL cDNA and support protein plasmids. A subtype A full length DNA copy and the four related support genes (N, P, L and M2), belonging to strain 14/1 (158), expressed in low copy plasmids, were used as templates for SDM and ligation reactions.

Site-directed mutagenesis. Subtype B amplicons, generated by both A and B type matching primers, were employed as megaprimers for SDM (188). These DNA sequences were able to join at both their extremities to the A type genome, resulting, after PFU polymerase reactions, in full length DNA copies including the B type sequence (Figure 23). SDM products were then run on agarose gel to confirm the success of the reaction (Figure 24).

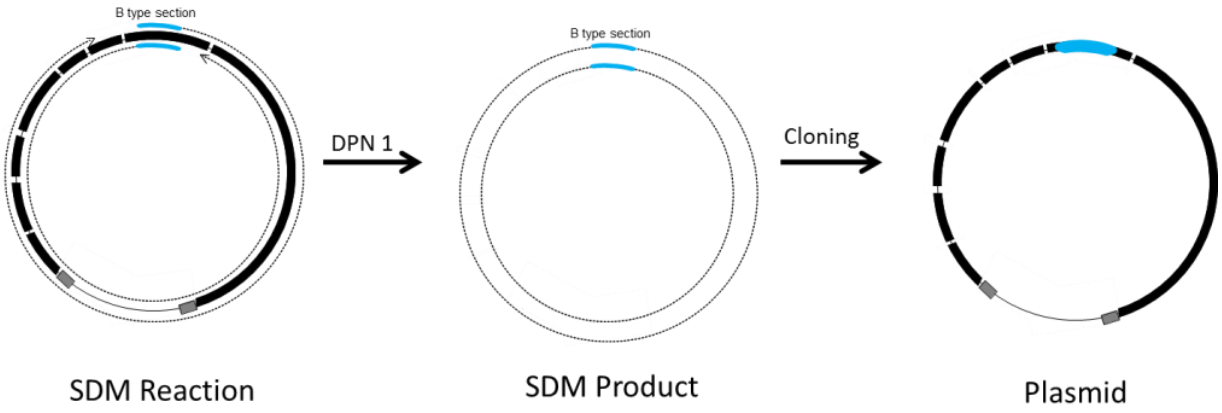

Figure 23: Schematic SDM and cloning process

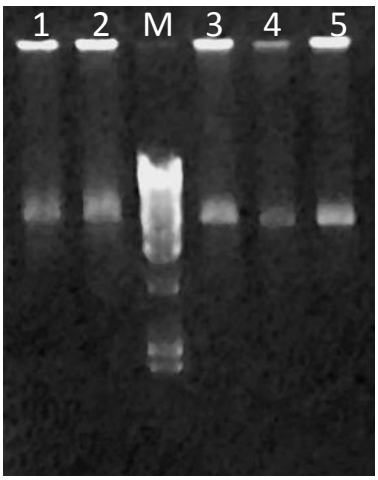

Figure 24: Examples of positive SDM reactions (1,2,3,4 and 5) observed on agarose gel, proving the successful of the PCR cycle. M is marker

Cut and Ligations protocols. Ligations protocols were employed when SDMs did not succed. Sal I, Xho I, EcoR V RE were mainly used for this purpose. Sticky ended ligations were more commonly used due to their high success rate: RE sites were introduced in the A type cDNAs by SDM reactions performed with enzyme sites adding primers. Sequences to be inserted were then constructed by designing specific primers including complementary cut sequences. After digestion of the artificially added sites, ligations were performed by T7 DNA ligases (Fermentas) (158, 188). This protocol is summarized in Figure 25.

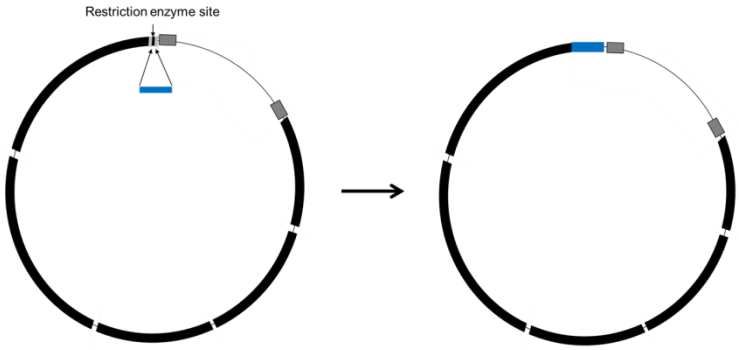

Figure 25: Schematic Ligation process

Cloning in *E.coli.* SDMs and ligations products were cloned in *E.coli* stb12 cells (Invitrogen). In a few cases even competent cells, derived from a commercial line, were prepared in our laboratory using calcium chloride protocols and employed for transformation (188). During the cloning process, amplicons were adsorbed into bacteria, methylated and transformed in bacterial plasmids, resulting in stabilization and replication of the template (188). Bacterial growth was performed at room temperature. Furthermore prior to SDMs cloning, products were treated with DPN 1. This enzyme destroys methylated constructs and is used to eliminate any traces of the original plasmid used for preparation of PCR mix. Selection of the right construct was performed by specific B type PCRs (Figure 26) or specific junction PCRs. Correct plasmids were than extracted using commercial kits (Qiagen). As deletions can often occur during cloning, specific cuts using REs were finally made on the extracted product, using agarose gel staining to assure the presence of the whole plasmid.

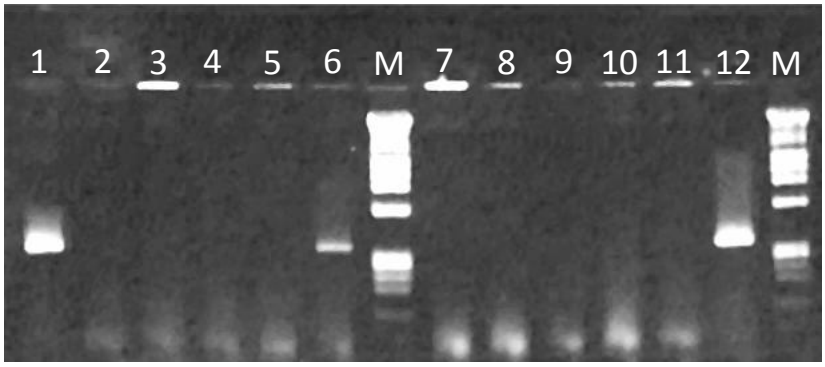

Figure 26: Example of colony screening after cloning in E.coli, with subtype B specific primers; only samples 1, 6 and 12 are positive

Sequencing. Constructs were then sequenced as definitive proof of the changes made and to exclude the presence of undesirable mutations. Products were purified by Exonuclease 1 and Alkaline Phosphatase treatment and sent to commercial sequencing companies (Cogenics). Sequence Analysis was carried out using Chromas, Bioedit and Gene Runner softwares.

5.4 RESULTS

Construction of subtype B FL cDNA. The SDM technique was successful for the exchange of several DNA subtype A sections to subtype B, allowing, in several steps, more than half the genome (about 7.7 kb) to be replaced (Figure 27). First of all, it was possible to replace a section of about 2 kb corresponding to N and P genes. Then almost all of the M gene and a short section of L and also the F gene and the beginning of the M2 were exchanged into the B type. Finally a further section of 2 kb was replaced in the L gene. However at this stage it proved impossible to add further subtype B genome sections. SDM reactions gave no further products and transformation did not lead to clones with the intended sequence. In particular it appeared that certain genome sections including the second half of the polymerase gene were not being tolerated by the *E.coli*. Bacteria showed unstable growth rates. Even when SDM reactions were successful, colonies were growing very slowly and dying very easily or sometimes they were not growing at all

As an alternative approach, it was decided to clone half genome sections, in the hope that they might be accepted more easily by the bacteria.

These were each derived from the last full length cDNA to be modified (Figure 27.6) using RE cuts followed by ligation steps. In this way two different half genome (HG) cDNAs were constructed, a leader and a trailer one (Figure 27.7). Further SDMs were then performed on these constructs. As a result, the leader HG was completely exchanged, resulting in 8,4 kb of B type sequence successfully cloned. Unfortunately no improvements were possible for trailer HG where the L gene second half continued to cause problems. At this stage, cut and ligation steps were used in order to add the remaining B type genome to the leader HG cDNA, resulting in a construct including B type sequences from the leader to 12100 kb (Figure 27.9). No further improvements were possible, despite several attempts. This included the use of new developed competent cells which were employed at this stage, but without any improvement.

Construction of single gene subtype B DNA copies. Cloning of M2 was achieved using SDM on the subtype A encoding M2 plasmid. N and P were instead constructed by ligation of the genes into plasmids, while no progresses were made for the complete subtype B L gene. Only partial L gene sequences have been cloned, specifically the first 4 kb, as several attempts to clone the full polymerase always resulted in trailer deleted constructs

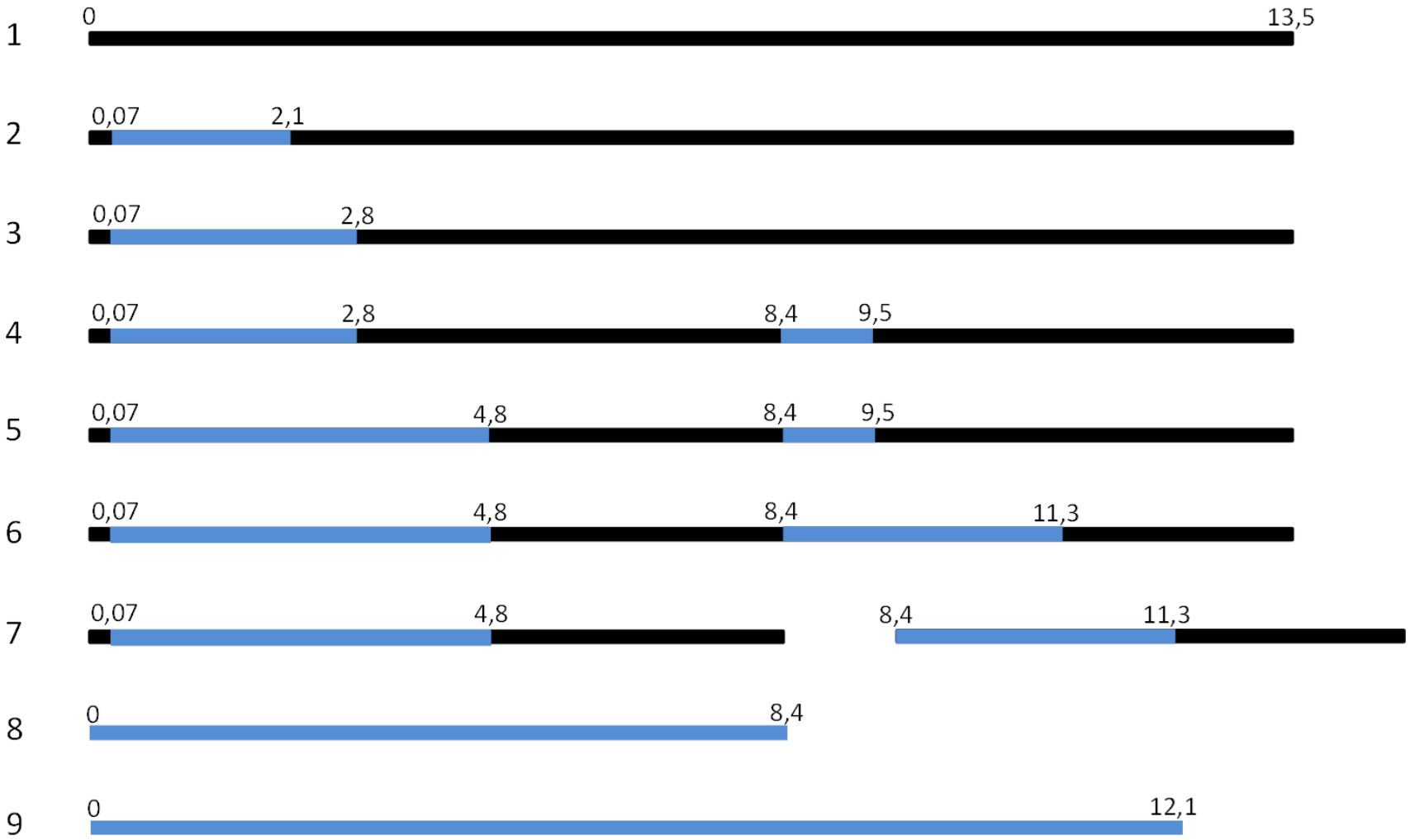

Figure 27: diagram showing the B type sequences, in blue, introduced in the A type FL cDNA by SDM (1 to 6). Half genome plasmid were constructed (7), allowing cloning of about 85 % of the B subtype genome (8 – 9).

5.5 DISCUSSION

As expected the development of a RG system for subtype B proved to be very challenging. Previous experiences setting up the A subtype RG system revealed the construction of FL cDNA to be critical (158). For this reason several strategies were employed to facilitate the cloning process, as using very tolerant competent cells both commercial and new developed ones (100, 197), low temperature growth for bacterial culture and low copy plasmids (188). Furthermore SDMs (230) were employed at first as it was thought to be more likely to succed than cut and ligation protocols. This technique allowed us to exchange more than 50% of the A type genome into the B type. However, after that, cloning attempts proved unsuccessful: in particular bacteria showed unstable growth rates. Even when SDM reactions were successful, colonies were growing very slowly and dying very easily or sometimes they were not growing at all. As mutations can occur during PCR based processes (102), changes in the kanamycin gene included in the plasmid were suspected. This might have explained the bacterial growth difficulties faced in the last stages. Sequence analysis were then performed on this region, but without showing any mutations. All these signs suggested that *E.coli* was unable to tolerate some of the viral sequences, since perhaps they might have encoded for toxic peptides, able to interfere with bacteria metabolic processes (188).

Furthermore the more B type sequences were cloned and the more these problems were increased. In order to try to address this critical situation, half genome plasmids were than constructed. It was speculated that plasmids containing only a portion of the viral genome could be better tolerated by the

bacterium than the mixed A and B subtype full length construct. This suggestion appeared to be correct as it was possible, at least with the leader half genome plasmid, to replace B type sequences until 8400 kb by SDM and then elonging it until 12100 kb by ligation steps. This resulted in more than 85 % of B type genome cloned. Unfortunately after that the same bacterial stability problems were faced again.

Three of the four required support gene plasmids were easily obtained, M2 by SDM and N and P by ligations. However it was not possible to clone the whole L gene (almost 6 kb). This highlighted once again how simple it is to clone small sections - N, P and M2 are no longer than 1300 kb each - compared to large sequences such as L or the full length cDNA. Furthermore attempts to clone the L gene resulted in cDNA deletion of the 5' end gene portion. That leaded to the idea that the trailer part of the genome, corresponding to the last part of the L gene, could be particularly toxic for bacteria. In support of this, there is evidence gained by the unsuccessful results in trying to exchange or elongate the trailer half genome plasmid.

In conclusion, this chapter describes the attempt to construct a full length cDNA for subtype B. More than 85% of the subtype B genome was cloned in a plasmid and three support plasmids, N, P and M2 were obtained. However bacteria instability problems, due to viral sequence intolerance, did not allowed us to obtain all the full cDNAs critical for the RG system. In the future further attempts will be made, trying to improve or change some of the materials and methods used for this work. For example more tolerant *E.coli* might be developed, and more stable plasmid vectors could be employed (188). Finally different cloning methods should be considered (196, 207), e.g. cloning in vaccinia virus, as reported for *Infectious bronchitis virus* RG systems, might be a valid alternative to bacteria cloning (29).

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Since RG was first applied to AMPV (92, 158) different reports have investigated the effects of single and multiple genomic mutations (23, 160, 163) and of gene deletions (133, 243) and insertions (92, 136) on viral biology. In this study we have tested AMPV as a vector for an extra homologus sequence (Chapter 2), for a reporter gene (Chapters 3 and 4) and for other viruses genes (Chapter 4). Finally an attempt to extend RG studies to AMPV subtype B was described in Chapter 5.

In Chapter 2 an AMPV DNA copy was modified to accept a homologous M2 sequence inside the G gene. We proposed that rescued virus could function as a positive control for an AMPV diagnostic RT nested PCR. RT-nested-PCR is a flexible virus detection technique of high sensitivity which requires the presence of the targeted RNA for positive detections. However, such sensitivity leads to potential risk of false test positives due to contamination with residual environmental nucleic acids. For this reason, the inclusion of a negative control appears to be critical (166). On the other hand a positive control is also required to avoid the occurrence of false negative results due to failure of the polymerase reaction (144). Standard controls derived from viral isolates have usually been employed, however these can enhance risk of false positives, as they can be themselves a source of contamination. The novel control virus described here removes the need to choose between conflicting demands. As for conventional AMPV control standards, the presence of correct bands confirms that all stages of the detection system, namely RNA extraction, reverse transcription and PCR, are functional. In addition, the increased size of amplicons immediately identifies instances where sample test positives result from contamination with the novel control. A potential problem with adopting such a live control virus in some regions might have been its genetically modified nature. However the microwave treatment effectively destroys virus

infectivity. Hence a control was developed which does not present any infection risks so can be used safely in all laboratory situations. The methodology is likely to be similarly useful for preparation of positive controls for the many RT-PCR tests used for detection of other RNA viruses, both avian (38, 85, 204) and non-avian (10, 27, 72). The addition of a complete foreign gene to subtype A AMPV has already been demonstrated (137) hence it will be a routine matter to similarly add sequence from viruses such as those listed, irrespective of their role in host viruses. Again the foreign virus sequence would need modification to enable amplicon size differentiation but problems of its incorporation into AMPV anywhere within the regions coding for the nonessential SH or G genes (158) would not be expected to curtail virus replication. Furthermore, while the inserted small RNA sections would be highly unlikely to produce hazardous viruses, this risk would be entirely eliminated using the described microwave irradiation procedure. This would make the approach very suitable for generating controls for frequently used RT-PCR tests detecting hazardous viruses, including influenza and HIV.

In order to assess the AMPV capacity to behave as a vector for foreign genes, seven strains of AMPVs were constructed each one carrying GFP in a different intergenic position (Chapter 3). AMPV tolerated GFP in all positions and good viral titres were generated in every case, except for one recombinant with insertion of GFP between N and P. This might be explained by an altered N/P ratio due to the addition of GFP between the two proteins as suggested by similar studies using VSV recombinants (232). N and P are in fact two essential components of the ribonuclear complex (RNP), which is critical for genome replication and transcription. Studies on VSV have shown the molar ratio between these two proteins to be critical for RNP functionality and an insertion

in that position seems to significantly alter this ratio, causing reduced virus production (106, 174) and this might explain the low titre.

GFP expression was confirmed for all the viruses and this complies with previous AMPV studies (92, 136). However, when this was quantified, huge differences were found among recombinants. GFP proved to be more highly expressed when inserted firstly in the N-P position, secondly in SH-G and then in G-L, while insertions in M-F, F-M2 and M2-SH lead to very low synthesis of the protein. This surprisingly was in contrast with the accepted theory regarding transcription processes in NNS RNA viruses, where gene transcription and expression are regulated by gene position with respect to the polymerase single entry point at 3' end. In this type of virus 3' proximal genes appear to be the most transcribed while the distal 5' ones are the least, with a sequential and gradual decrease at every gene junction of 20 – 30 % in terms of transcription rate, due to polymerase dissociation (108, 233). Therefore, we expected to detect the best GFP expression efficiency in r1 and least in r7, with the other recombinants varying GFP expression according to the distance of the inserted gene from the 3' end. According to these findings, we speculated that a future AMPV vector should ideally have an extra gene introduced between N and P for an efficient protein expression. However the very low titre reached by this recombinant suggested severe viability issues, and for this reason an insertion between SH and G seemed the ideal solution for a future recombinant. This study was only a preliminary *in vitro* investigation, therefore experimental infections with animals should be critical in confirming and assessing the importance of the previous speculations *in vivo*. In a previous study, a recombinant GFP-AMPV was used to infect birds and resulted in antibodies detection against

the vectored protein (92), showing the good potential of AMPV based recombinants.

Chapter 4 describes the construction of several recombinants carrying foreign genes. AMPV not only tolerates large extra sequences of up to 3000 nucleotides, as in the case of the N + S1 recombinant, but is also able to replicate *in vitro* to high titres despite these insertions. This complies with similar evidences showed by other NNS single stranded RNA viruses (147, 192, 229). GFP recombinants were constructed to test our expression system, consisting of the addition of an extra transcriptional unit in AMPV. IBV recombinants were then constructed in order to test AMPV, as a vector, for recombinant vaccines. Both IBV S1 and N genes were inserted, as they have both proved to induce protective immunity (35, 199, 242). *In vivo* some of the recombinants gave partial protection, assessed by observing tracheal cilial motility after virulent challenge with IBV. Unfortunately replication in the respiratory tract appeared to be poor for almost all recombinants -except for 309/09, which however had the worst cilial performances-, and the serological responses against AMPV and IBV were largely absent. These finding suggests that even if AMPV readily accepts large and multiple genes in its genome, a deeper understanding of its biological properties is needed, before it can be seriously considered as a vector for recombinant vaccines.

It would be interesting in the future to extend the same type of study to other AMPV subtypes (Chapter 5). The economic importance of the B subtype appears to be equal, if not in some cases higher than to subtype A. Despite a small number of attempts, up to now no RG system has been reported to allow genetic manipulation of this viral subgroup. For this reason the development of a subtype B RG was attempted here. The construction of a full length DNA copy (cDNA) was attempted using site-directed mutagenesis and ligation techniques

and this resulted in more than 85 % of B type genome being cloned. Furthermore, as for negative-stranded viruses the formation of the ribonuclear complex (RNP) is essential to start viral activities, RNP support proteins genes cDNAs were also constructed for N, P and M2. On the other hand, cloning of the L gene did not succeed. Both full length cDNA and L cDNA faced severe development problems relating to construct instability and unsuccessful cloning due to probable bacteria intolerance. For example SDM and ligation reactions would not consistently work and when they were successful, transformation in *E.coli* resulted in delayed or absent bacterial growth or premature bacterial death. In order to construct the B subtype RG in the future, different approaches should be applied for this aim, such as: the use of more tolerant *E.coli* or more stable plasmid (188). Furthermore different cloning methods should be considered (196, 207), e.g. cloning in vaccinia virus, as reported for *Infectious bronchitis virus* RG systems, might be a valid alternative to bacteria cloning (29).

In conclusion, AMPV subtype A has proved to be very tolerant in accepting extra sequences both within non-essential genes and within intergenic positions. Extra nucleotide sequences were conserved through several passages, viral viability appeared to be not affected *in vitro* despite insertions and expression of foreign genes was demonstrated in all experiments. In two cases, viruses have been tested as candidate vaccines in chickens against IBV showing promising results. Unfortunately it was not possible to extend these studies to subtype B. However the experience gained and the constructs obtained will hopefully be useful in the future to achieve these targets.

REFERENCES

1. **Al-Ankari, A. R., J. M. Bradbury, C. J. Naylor, K. J. Worthington, C. Payne-Johnson, and R. C. Jones.** 2001. Avian pneumovirus infection in broiler chicks inoculated with Escherichia coli at different time intervals. Avian Pathol **30:**257-267.

2. **Alexander, D. J., E. D. Borland, C. D. Bracewell, N. J. Chettle, R. E. Gough, and S. A. Lister, Wyeth, P.J.** 1986. A preliminary report of investigations into turkey rhinotracheitis in Great Britain. State Veterinary Journal **40:**161-169.

3. **Ali, A., and D. L. Reynolds.** 2000. A multiplex reverse transcription-polymerase chain reaction assay for Newcastle disease virus and avian pneumovirus (Colorado strain). Avian Dis **44:**938-943.

4. **Ali, A., and D. L. Reynolds.** 1999. A reverse transcription-polymerase chain reaction assay for the detection of avian pneumovirus (Colorado strain). Avian Dis **43:**600-603.

5. **Alkahalaf, A. N., L. A. Ward, R. N. Dearth, and Y. M. Saif.** 2002. Pathogenicity, transmissibility and tissue distribution of avian pneumovirus in turkey poults. Avian Diseases 650-659.

6. **Andral, B., C. Louzis, E. Edlinger, J. A. Newman, D. Toquin, and G. Bennejean.** 1985. Respiratory disease (rhinotracheitis) in turkeys in Brittany, France, 1981-1982. II. Laboratory findings. Avian Dis **29:**35-42.

7. **Anon.** 1985. (Preliminary report from the British Veterinary Poultry Association) Turkey rhinotracheitis of unknown ateology in England and Wales. Vet Rec **117:**653-654.

8. **Arns, C. W., and H. M. Hafez.** 1995. Presented at the 44th Western Poultry Disease Conference Sacramento, California, USA.

9. **Aung, Y. H., M. Liman, U. Neumann, and S. Rautenschlein.** 2008. Reproducibility of swollen sinuses in broilers by experimental infection with avian metapneumovirus subtypes A and B of turkey origin and their comparative pathogenesis. Avian Pathol **37:**65-74.

10. **Bagnarelli, P., S. Menzo, A. Manzin, M. Giacca, P. E. Varaldo, and M. Clementi.** 1991. Detection of human immunodeficiency virus type 1 genomic RNA in plasma samples by reverse-transcription polymerase chain reaction. J Med Virol **34:**89-95.

11. **Baxter-Jones, C., J. K. Cook, J. A. Frazier, M. Grant, R. C. Jones, A. P. Mockett, and G. P. Wilding.** 1987. Close relationship between TRT virus isolates. Vet Rec **120:**562.

12. **Bayon-Auboyer, M. H., C. Arnauld, D. Toquin, and N. Eterradossi.** 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. J Gen Virol **81:**2723-2733.

13. **Bayon-Auboyer, M. H., V. Jestin, D. Toquin, M. Cherbonnel, and N. Eterradossi.** 1999. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Arch Virol **144:**1091-1109.

14. **Bell, I. G., and D. J. Alexander.** 1990. Failure to detect antibody to turkey rhinotracheitis virus in Australian poultry flocks. Aust Vet J **67:**232-233.

15. **Bennett, R. S., B. McComb, H. J. Shin, M. K. Njenga, K. V. Nagaraja, and D. A. Halvorson.** 2002. Detection of avian pneumovirus in wild Canada (Branta canadensis) and blue-winged teal (Anas discors) geese. Avian Dis **46:**1025-1029.

16. **Bennett, R. S., J. Nezworski, B. T. Velayudhan, K. V. Nagaraja, D. H. Zeman, N. Dyer, T. Graham, D. C. Lauer, M. K. Njenga, and D. A. Halvorson.** 2004. Evidence of avian pneumovirus spread beyond Minnesota among wild and domestic birds in central North America. Avian Dis **48:**902-908.

17. **Bhattacharjee, P. S., C. J. Naylor, and R. C. Jones.** 1994. A simple method for immunofluorescence staining of tracheal organ cultures for the rapid identification of infectious bronchitis virus. Avian Pathol **23:**471-480.

18. **Bidin, Z., Z. Stanisic, S. Curic, I. Rusac, and M. Mikec, 1990. .** 1990. Rinotracheitis in turkeys. Clinical picture and specific serum antibody findings in the first report case of the disease in Yugoslavia. Vet Arhiv.

19. **Botchkov, Y., A. Borisov, V. Irza, T. Okovytaya, E. Ovchinnikova, G. Batchenko, T. Manin, L. Scherbakova, V. Drygin, and E. Guseva.** 2002. Presented at the 11th European Poultry Conference, Bremen, Germany, 6-10 Sept 2002.

20. **Britton, P., P. Green, S. Kottier, K. L. Mawditt, Z. Penzes, D. Cavanagh, and M. A. Skinner.** 1996. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. J Gen Virol **77 (Pt 5):**963-967.

21. **Broor, S., and P. Bharaj.** 2007. Avian and Human Metapneumovirus. Ann. N.Y. Acad. Sci. **1102:**66 - 85.

22. **Brown, P. A., C. Lupini, E. Catelli, J. Clubbe, E. Ricchizzi, and C. J. Naylor.** 2011. A single polymerase (L) mutation in avian metapneumovirus increased virulence and partially maintained virus viability at an elevated temperature. Journal of General Virology **92:**346-354.

23. **Brown, P. A., Lupini C., Catelli E., Clubbe J., Ricchizzi E., Naylor C.J.** 2011. A single polymerase (L) mutation in avian metapneumovirus increased virulence and partially maintained virus viability at an elevated temperature. Journal of General Virology **92:**346-354.

24. **Buys, S. B., and J. H. Du Prees.** 1980. A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. Turkeys **28:**36

25. **Buys, S. B., J. H. du Preez, and H. J. Els.** 1989. The isolation and attenuation of a virus causing rhinotracheitis in turkeys in South Africa. Onderstepoort J Vet Res **56:**87-98.

26. **Cadman, H. F., P. J. Kelly, R. Zhou, F. Davelaar, and P. R. Mason.** 1994. A serosurvey using enzyme-linked immunosorbent assay for antibodies against poultry pathogens in ostriches (Struthio camelus) from Zimbabwe. Avian Dis **38:**621-625.

27. **Callens, M., and K. De Clercq.** 1997. Differentiation of the seven serotypes of foot-and-mouth disease virus by reverse transcriptase polymerase chain reaction. J Virol Methods **67:**35-44.

28. **Capua, I., Z. Minta, E. Karpinska, K. Mawditt, P. Britton, D. Cavanagh, and E. Gough.** 1999. Co-circulation of four types of infectious bronchitis(793/B, 624/I, B1648 and Massachusetts). Avian Pathology **28:**587–592.

29. **Casais, R., V. Thiel, S. G. Siddel, D. Cavanagh, and P. Britton.** 2001. Reverse Genetics System for Avian Coronavirus Infectious Bronchitis Virus. Journal of Virology **75:**12359 - 12369.

30. **Catelli, E., M. Cecchinato, C. E. Savage, R. C. Jones, and C. J. Naylor.** 2006. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. Vaccine **24:**6476-6482.

31. **Catelli, E., M. Cecchinato, C. E. Savage, R. C. Jones, and C. J. Naylor.** 2006. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. Vaccine **24:**6476-6482.

32. **Catelli, E., J. K. A. Cook, J. Chesher, S. J. Orbell, M. A. Woods, W. Baxendale, and M. B. Huggins.** 1998. The use of virus isolation,histopathology and immunoperoxidase techniques to study the dissemination of a chicken isolate of avian pneumovirus in chickens. Avian Pathol **27:**632-640.

33. **Catelli, E., M. A. De Marco, M. Delogu, C. Terregino, and V. Guberti.** 2001. Serological evidence of avian pneumovirus infection in reared and free-living pheasants. Vet Rec **149:**56-58.

34. **Catelli, E., C. Lupini, M. Cecchinato, E. Ricchizzi, P. Brown, and C. J. Naylor.** 2010. Field avian metapneumovirus evolution avoiding vaccine induced immunity. Vaccine **28:**916-921.

35. **Cavanagh, D.** 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. **38:**281-297.

36. **Cavanagh, D., P. J. Davis, J. H. Darbyshire, and R. W. Peters.** 1986. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J Gen Virol **67 (Pt 7):**1435-1442.

37. **Cavanagh, D., M. M. Ellis, and J. K. A. Cook.** 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathology **26:**63-74.

38. **Cavanagh, D., K. Mawditt, P. Britton, and C. J. Naylor.** 1999. Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Pathology **28:**593-605.

39. **Cecchinato, M., E. Catelli, C. Lupini, E. Ricchizzi, J. Clubbe, M. Battilani, and C. J. Naylor.** 2010. Avian metapneumovirus (AMPV) attachment protein involvement in probable virus evolution concurrent with mass live vaccine introduction. Vet Microbiol **146:**24-34.

40. **Cecchinato M., L. C., Mondin A., Muñoz Pogoreltseva O.S., Listorti V.,Catelli E.** 2011. Development of Real Time RT-PCR Tests for Detection andDifferentiation of Avian Metapneumovirus. 17th World Veterinary PoultryCongress – Proceedings, Cancun, México, 14-16 August 2011**:**334-341.

41. **Cecchinato, M. L., C. Mondin, A. Muñoz Pogoreltseva, O.S. Listorti, V. Catelli, E.** 2011. Development of Real Time RT-PCR Tests for Detection andDifferentiation of Avian Metapneumovirus. 17th World Veterinary PoultryCongress – Proceedings, Cancun, México, 14-16 August 2011**:**334-341.

42. **Clark, S.** 1998. A Review of Avian Pneumovirus. Proceedings of the Roche Avian Pneumovirus Workshop. June 30-1 July, St Cloud, Minnesota, USA**:**21-24.

43. **Clubbe, J., and C. J. Naylor.** 2011. Avian metapneumovirus M2:2 protein inhibits replication in Vero cells: modification facilitates live vaccine development. Vaccine **29:**9493-9498.

44. **Clubbe, J. N., C.J.** 2011. Avian metapneumovirus M2:2 protein inhibits replication in Vero cells: modification facilitates live vaccine development. Vaccine **29:**9493-9498.

45. **Collins, M. S., R. E. Gough, and D. J. Alexander.** 1993. Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. Avian Pathology **22:**469-479.

46. **Collins, M. S., R. E. Gough, S. A. Lister, N. Chettle, and R. Eddy.** 1986. Further characterisation of a virus associated with turkey rhinotracheitis. Vet Rec **119:**606.

47. **Conzelmann, K.** 2003. Reverse Genetics of Mononegavirales. Current topics in microbiology and immunology**:**1 - 42.

48. **Conzelmann, K., and G. Meyers.** 1996. Genetic engineering of animal RNA viruses. Trends in Microbiology **4:**386 - 393.

49. **Cook, J. K.** 2000. Avian pneumovirus infections of turkeys and chickens. Vet J **160:**118-125.

50. **Cook, J. K.** 2000. Avian rhinotracheitis. Rev Sci Tech **19:**602-613.

51. **Cook, J. K., and D. Cavanagh.** 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathol **31:**117-132.

52. **Cook, J. K., J. Chesher, F. Orthel, M. A. Woods, S. J. Orbell, W. Baxendale, and M. B. Huggins.** 2000. Avian pneumovirus infection of laying hens: experimental studies. Avian Pathol **29:**545-556.

53. **Cook, J. K., M. M. Ellis, and M. B. Huggins.** 1991. The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. Avian Pathol **20:**155-166.

54. **Cook, J. K., M. B. Huggins, S. J. Orbell, K. Mawditt, and D. Cavanagh.** 2001. Infectious bronchitis virus vaccine interferes with the replication of avian pneumovirus vaccine in domestic fowl. Avian Pathol **30:**233-242.

55. **Cook, J. K., M. B. Huggins, S. J. Orbell, and D. A. Senne.** 1999. Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA. Avian Pathology **28:**607-617.

56. **Cook, J. K., M. B. Huggins, M. A. Woods, S. J. Orbell, and A. P. Mockett.** 1995. Protection provided by a commercially available vaccine against different strains of turkey rhinotracheitis virus. Vet Rec **136:**392-393.

57. **Cook, J. K., B. V. Jones, M. M. Ellis, L. Jing, and D. Cavanagh.** 1993. Antigenic differentiation of strains of turkey rhinotracheitis virus using monoclonal antibodies. Avian Pathology **22:**257-273.

58. **Cook, J. K., S. Kinloch, and M. M. Ellis.** 1993. In vitro and in vivo studies in chikens and turkeys on strains of turkey rhinotracheitis virus isolated from the two species Avian Pathology **22:**157-170.

59. **Cook, J. K., S. J. Orbell, M. A. Woods, and M. B. Huggins.** 1996. A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). Veterinary Record **138:**178 - 180.

60. **Cook, J. K. A., and M. M. Ellis.** 1990. Attenuation of turkey rhinotracheitis virus by alternative passage in embryonated chicken eggs and tracheal organ cultures. Avian Pathol **19**.

61. **Cook, J. K. A., M. M. Ellis, and M. B. Huggins.** 1991. The pathogenesis of Turkey rhinotracheistis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. Avian Pathology **20:**155-156.

62. **Cook, J. K. A., H. C. Holmes, P. M. Finney, C. A. Dolby, M. M. Ellis, and M. B. Huggins.** 1989. A Live Attenuated Turkey Rhinotracheitis Virus-Vaccine .2. The Use of the Attenuated Strain as an Experimental Vaccine. Avian Pathology **18:**523-534.

63. **Cook, J. K. A., F. Orthel, S. Orbell, M. A. Woods, and M. B. Huggins.** 1996. An experimental turkey rhinotracheitis (TRT) infection in breeding turkeys and the prevention of its clinical effects using live-attenuated and inactivated TRT vaccines. Avian Pathology **25:**231-243.

64. **Coswig, L. T., M. B. Santos, H. M. Hafez, H. L. Ferreira, and C. W. Arns.** 2010. Propagation of Avian Metapneumovirus Subtypes A and B Using Chicken Embryo-Related and Other Cell Systems. J Virol Methods.

65. **Cubillos, A., J. Ulloa, V. Cubillos, and J. K. A. Cook.** 1991. Characterization of Strains of Infectious-Bronchitis Virus Isolated in Chile. Avian Pathology **20:**85-99.

66. **Decanini, E. L., E. C. Miranda, and F. X. Le Gros.** 1991. Presented at the 40th Western Poultry Disease Conference Acapulco, Mexico.

67. **Delogu, M., M. A. De Marco, E. Catteli, M. Cecchinato, R. L. Sperati, P. Pesente, and C. Franciosi.** 2004. Presented at the IV. International symposium on avian corona and pneumovirus infections., Rauischholzhausen, Germany, 20-23 June 2004.

68. **Dimmock, N. J., A. J. Easton, and K. N. Leppard.** 2007. The Process of infection: IIIB. Gene expression and its regulation in RNA viruses. Introduction to modern virology, Sixth edition, Blackwell Publishing **10:**167 - 171.

69. **Drouin, P., J. Y. Toux, and J. P. Picault.** 1985. Le syndrome infectieux de la grosse tete chez l'espèce poule. L'aviculteur **460**.

70. **Easton, A. J., J. B. Domachowske, and H. F. Rosenberg.** 2004. Animal pneumoviruses: molecular genetics and pathogenesis. Clin Microbiol Rev **17:**390-412.

71. **Elhafi, G., C. J. Naylor, C. E. Savage, and R. C. Jones.** 2004. Microwave or autoclave treatments destroy the infectivity of infectious bronchitis virus and avian pneumovirus but allow detection by reverse transcriptase-polymerase chain reaction. Avian Pathol **33:**303-306.

72. **Ellis, J. S., D. M. Fleming, and M. C. Zambon.** 1997. Multiplex reverse transcription-PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996. J Clin Microbiol **35:**2076-2082.

73. **Engstrom, B., G. Czifra, and D. Cavanagh.** 2000. Presented at the Third International Symposium on Turkey Diseases, Berlin, 14-17 June 2000.

74. **Eterradossi, N., D. Toquin, M. Guittet, and G. Bennejean.** 1995. Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. Veterinary Medicine **42:**175-186.

75. **Fabris, G., and P. N. D'aprile.** 1990. Rinotracheite infettiva del tacchino: osservazioni sul campo ed indagini di laboratorio. Zootecnica International **I:**36-40.

76. **Fearns, C., M. E. Peeples, and P. L. Collins.** 1997. Increased expression of the N protein of respiratory syncytial virus stimulates minigenome replication but does not alter the balance between the synthesis of mRNA and antigenome. Virology **236:**188 - 201.

77. **Follett, E. A., C. L. Pringle, and T. H. Pennington.** 1975. Virus development in enucleate cells: echovirus, poliovirus, reovirus, respiratory syncytial virus and Semliki Forest virus. Journal of General Virology **26:**183 - 196.

78. **Frangipani, J. A., and C. Jungback.** 2004. Immunogenicity testing of vaccines against Avian Infectious Bronchitis - Comparison of several methods to demonstrate experimental infection. Proceedings IV International Symposium on Avian Corona- and Pneumovirus Infections**:**202 - 212.

79. **Ganapathy, K., P. Cargill, E. Montiel, and R. C. Jones.** 2005. Interaction between live avian pneumovirus and Newcastle disease virus vaccines in specific pathogen free chickens. Avian Pathol **34:**297-302.

80. **Ganapathy, K., R. C. Jones, and J. M. Bradbury.** 1998. Pathogenicity of in vivo-passaged Mycoplasma imitans in turkey poults in single infection and in dual infection with rhinotracheitis virus. Avian Pathol **27:**80-89.

81. **Ganapathy, K., V. Todd, P. Cargill, E. Montiel, and R. C. Jones.** 2006. Interaction between a live avian pneumovirus vaccine and two different Newcastle disease virus vaccines in broiler chickens with maternal antibodies to Newcastle disease virus. Avian Pathol **35:**429-434.

82. **Gharaibeh, S. M., and G. R. Algharaibeh.** 2007. Serological and molecular detection of avian pneumovirus in chickens with respiratory disease in Jordan. Poult Sci **86:**1677-1681.

83. **Ghorashi, S. A., F. Ghasemi, and R. Salehi Tabar.** 2007. Direct detection of IBV, APV and NDV in clinical respiratory speciemens by a multiplex reverse transcription-PCR assay. Proceedings of the 15th World Poultry Congress, 2007 Beijing, China Abstrat Book**:**412.

84. **Giraud, P., G. Bennejean, M. Guittet, and D. Toquin.** 1986. Turkey rhinotracheitis in France: preliminary investigations on a ciliostatic virus. Vet Rec **119:**606-607.

85. **Gohm, D. S., B. Thur, and M. A. Hofmann.** 2000. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. Avian Pathol **29:**143-152.

86. **Gough, R. E., and M. S. Collins.** 1989. Antigenic relationships of three turkey rhinotracheitis viruses. Avian Pathology **18:**227-238.

87. **Gough, R. E., M. S. Collins, W. J. Cox, and N. J. Chettle.** 1988. Experimental infection of turkeys, chickens, ducks, geese, guinea fowl, pheasants and pigeons with turkey rhinotracheitis virus. Vet Rec **123:**58-59.

88. **Gough, R. E., S. E. Drury, E. Aldous, and P. W. Laing.** 2001. Isolation and identification of avian pneumovirus from pheasants. Vet Rec **149:**312.

89. **Gough, R. E., and R. C. Jones.** 2008. Avian Metapneumoviruses, 12 ed. Blackwell Publishing, Ames, Iowa, USA.

90. **Gough, R. E., and J. C. Pedersen.** 2008. Avian Metapneumovirus. A laboratoty manual for the identification, and characterization of avian pathogens, Fifth Edition, The American Association of Avian Pathologists**:**142 - 145.

91. **Govindarajan, D.** 2005. Reverse Genetics of Avian Metapneumovirus. Doctor of Philosophy Thesys, Faculty of the Graduate School of the University of Maryland, College Park.

92. **Govindarajan, D., U. J. Buchholz, and S. K. Samal.** 2006. Recovery of avian metapneumovirus subgroup C from cDNA: cross-recognition of avian and human metapneumovirus support proteins. J Virol **80:**5790-5797.

93. **Goyal, S. M., S. J. Chiang, A. M. Dar, K. V. Nagaraja, D. P. Shaw, D. A. Halvorson, and V. Kapur.** 2000. Isolation of avian pneumovirus from an outbreak of respiratory illness in Minnesota turkeys. J Vet Diagn Invest **12:**166-168.

94. **Grant, M., C. Baxter-Jones, and G. P. Wilding.** 1987. An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. Vet Rec **120:**279-280.

95. **Guionie, O., D. Toquin, E. Sellal, S. Bouley, F. Zwingelstein, C. Allee, S. Bougeard, S. Lemiere, and N. Eterradossi.** 2007. Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. J Virol Methods **139:**150-158.

96. **Hafez, H. M.** 1993. The role of pneumovirus in swollen head syndrome of chickens: review. Arch.Geflugelkde**:**181-185.

97. **Hafez, H. M., J. Emele, and H. Woernle.** 1990. Turkey rhinotracheitis:serological flock profiles and economic parameters and treatment trials using Enrofloxacin (Baytril). Zeitschr Gebiete Veterin **45:**111-114.

98. **Hafez, H. M., M. Hess, C. Prusas, C. J. Naylor, and D. Cavanagh.** 2000. Presence of avian pneumovirus type A in continental Europe during the 1980s. J Vet Med B Infect Dis Vet Public Health **47:**629-633.

99. **Hafez, H. M., and H. Woernle.** 1989. Turkey rhinotracheitis, serological results in Baden-Wurttemnberg. Tierarztliche-Umschau **44:**476-480.

100. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmids. J Mol Biol. **166:**557-580.

101. **Heffels-Redman, U., U. Neuman, S. Braune, J. K. A. Cook, and J. Pruter.** 1998. Serological evidence for sucseptibility of seagulls to avian pneumovirus (APV) infection, Rauischholshausen, Germany.

102. **Herlitze, S., and M. Koenen.** 1990. A general and rapid mutagenesis method using polymerase chain reaction. Gene **91:**143-147.

103. **Hess, M., M. B. Huggins, and U. Heincz.** 2004. Hatchability, serology and virus excretion following in ovo vaccination of chickens with an avian metapneumovirus vaccine. Avian Pathol **33:**576-580.

104. **Hess, M., M. B. Huggins, R. Mudzamiri, and U. Heincz.** 2004. Avian metapneumovirus excretion in vaccinated and non-vaccinated specified pathogen free laying chickens. Avian Pathol **33:**35-40.

105. **Houadfi, E., A. Hamam, J. Vanmarcke, and J. K. A. Cook.** 1991. Presented at the 40th Western Poultry Disease Conference, Acapulco, Mexico.

106. **Howard, M., and G. W. Wertz.** 1989. Vescicular stomatitis virus RNA replication: a role for the NS protein. J Gen Virol **70:**2683 - 2694.

107. **Hu, H., J. P. Roth, C. N. Estevez, L. Zsak, B. Liu, and Q. Yu.** 2011. Generation and evaluation of a recombinant Newcastle disease virus expressing the glycoprotein (G) of avian metapneumovirus subgroup C as a bivalent vaccine in turkeys. Vaccine **29:**8624 - 8633.

108. **Iverson, L. E. R., J.K.** 1981. Localized attenuation and discontinuos synthesis during vescicular stomatitis virus transcription. Cell **23:**477-484.

109. **Jing, L., J. K. Cook, T. David, K. Brown, K. Shaw, and D. Cavanagh.** 1993. Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. Avian Pathol **22:**771-783.

110. **Jing, L., J. K. A. Cook, T. D. K. Brown, K. Shaw, C, and D. Cavanagh.** 1993. Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. Avian Pathol **22:**771-783.

111. **Jirjis, F. E., S. L. Noll, D. A. Halvorson, K. V. Nagaraja, and D. P. Shaw.** 2001. Immunohistochemical detection of avian pneumovirus in formalin-fixed tissues. J Vet Diagn Invest **13:**13-16.

112. **Jirjis, F. E., S. L. Noll, D. A. Halvorson, K. V. Nagaraja, E. L. Townsend, A. M. Sheikh, and D. P. Shaw.** 2000. Avian pneumovirus infection in Minnesota turkeys: experimental reproduction of the disease. Avian Dis **44:**222-226.

113. **Jirjis, F. F., S. L. Noll, D. A. Halvorson, K. V. Nagaraja, F. Martin, and D. P. Shaw.** 2004. Effects of bacterial coinfection on the pathogenesis of avian pneumovirus infection in turkeys. Avian Dis **48:**34-49.

114. **Jirjis, F. F., S. L. Noll, D. A. Halvorson, K. V. Nagaraja, and D. P. Shaw.** 2002. Pathogenesis of avian pneumovirus infection in turkeys. Vet Pathol **39:**300-310.

115. **Jones, R. C.** 1996. Avian pneumovirus infection: Questions still unanswered. Avian Pathol **25:**639-648.

116. **Jones, R. C., C. Baxter-Jones, C. E. Savage, D. F. Kelly, and G. P. Wilding.** 1987. Experimental infection of chickens with a ciliostatic agent isolated from turkeys with rhinotracheitis. Vet Rec **120:**301-302.

117. **Jones, R. C., C. Baxter-Jones, G. P. Wilding, and D. F. Kelly.** 1986. Demonstration of a candidate virus for turkey rhinotracheitis in experimentally inoculated turkeys. Vet Rec **119:**599-600.

118. **Jones, R. C., R. S. Khehera, C. J. Naylor, and D. Cavangh.** 1998. Presented at the International Symposium on Infectious Bronchitis and Avian Pneumovirus Infections in Poultry, Rauischholhausen, Germany.

119. **Jones, R. C., C. J. Naylor, A. al-Afaleq, K. J. Worthington, and R. Jones.** 1992. Effect of cyclophosphamide immunosuppression on the immunity of turkeys to viral rhinotracheitis. Res Vet Sci **53:**38-41.

120. **Jones, R. C., C. J. Naylor, J. M. Bradbury, C. E. Savage, K. Worthington, and R. A. Williams.** 1991. Isolation of a turkey rhinotracheitis-like virus from broiler breeder chickens in England. Vet Rec **129:**509-510.

121. **Jones, R. C., R. A. Williams, C. Baxter-Jones, C. E. Savage, and G. P. Wilding.** 1988. Experimental infection of laying turkeys with rhinotracheitis virus: distribution of virus in the tissues and serological response. Avian Pathology **17:**841-850.

122. **Juhasz, K., and A. J. Easton.** 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. J Gen Virol **75 (Pt 11):**2873-2880.

123. **Kapczynski, D. R., and H. S. Sellers.** 2003. Immunization of turkeys with a DNA vaccine expressing either the F or N gene of avian metapneumovirus. Avian Dis **47:**1376-1383.

124. **Kayali, G., E. J. Ortiz, M. L. Chorazy, K. V. Nagaraja, J. DeBeauchamp, R. J. Webby, and G. C. Gray.** 2011. Serologic evidence of avian metapneumovirus infection among adults occupationally exposed to Turkeys. Vector Borne Zoonotic Diseases **11:**1453 - 1458.

125. **Khehra, R. S., and R. C. Jones.** 1999. In vitro and in vivo studies on the pathogenicity of avian pneumovirus for the chicken oviduct. Avian Pathology **28:**257-262.

126. **Kingsbury, D. W. H., C. H. ; Murti, K. G. ;.** 1978. Intracellular metabolism of sendai virus nucleocapside. Virology **91:**86 - 94.

127. **Kong, B. W., L. K. Foster, and D. N. Foster.** 2006. Comparison of avian cell substrates for propagating subtype C avian metapneumovirus. Virus Research **116:**58-68.

128. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature **339:**237-238.

129. **Lai, C. J., B. Zhao, H. Hori, and M. Bray.** 1991. Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus. Proc. Natl. Acad. Sci., USA **88:**5139 - 5143.

130. **Lee, E., M. S. Song, J. Y. Shin, Y. M. Lee, C. J. Kim, Y. S. Lee, H. Kim, and Y. K. Choi.** 2007. Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. Virus Res **128:**18-25.

131. **Levine, S., R. Klaiber-Franco, and P. R. Paradiso.** 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J Gen Virol **68 (Pt 9):**2521-2524.

132. **Ling, R., A. J. Easton, and C. R. Pringle.** 1992. Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveals a gene order different from that of other pneumoviruses. J Gen Virol **73 (Pt 7):**1709-1715.

133. **Ling, R., S. Sinkovic, D. Toquin, O. Guionie, N. Eterradossi, and A. J. Easton.** 2008. Deletion of the SH gene from avian metapneumovirus has a greater impact on virus production and immunogenicity in turkeys than deletion of the G gene or M2-2 open reading frame. J Gen Virol **89:**525-533.

134. **Lou, F., H. Neetoo, J. Li, H. Chen, and J. Li.** 2011. Lack of correlation between virus barosensitivity and the presence of a viral envelope during inactivation of human rotavirus, vescicular stomatitis virus, and avian metapneumovirus by high pressure processing. Appl Environ Microbiol **77:**8538 - 8547.

135. **Lu, Y. S., Y. S. Shien, H. J. Tsai, C. S. Tseng, S. H. Lee, and D. F. Lin.** 1994. Swollen head syndrome in Taiwan-isolation of an avian pneumovirus and serological study. Avian Pathology **23:**169-174.

136. **Lupini, C., E. Catelli, M. Cecchinato, and C. J. Naylor.** 2008. Presented at the 7th International symposium on Turkey Diseases (in press) Hotel Steglitz International, Albrechtstraße 2, 12165 Berlin-SteglitzBerlin, Germany

137. **Lupini, C., E. Catelli, M. Cecchinato, and C. J. Naylor.** 2008. Presented at the 7th International symposium on Turkey Diseases, World veterinary Poultry Association (German Branch) ISBN 978-3-939902-96-6, p180. Construction of a green fluorescent protein (GFP) avian metapneumovirus (AMPV) recombinant lacking the small hydrophobic (SH) protein gene shows that giant syncytial formations are not a result of the reduction in genome size (2008), Berlin, May 19th to 21st 2008. Proceedings ISBN 978-3-939902-96-6.

138. **Maherchandani, S., D. P. Patnayak, C. A. Munoz-Zanzi, D. Lauer, and S. M. Goyal.** 2005. Evaluation of five different antigens in enzyme-linked immunosorbent assay for the detection of avian pneumovirus antibodies. J Vet Diagn Invest **17:**16-22.

139. **Majo, N., G. M. Allan, C. J. O'Loan, A. Pages, and A. J. Ramis.** 1995. A sequential histopathologic and immunocytochemical study of chickens, turkey poults, and broiler breeders experimentally infected with turkey rhinotracheitis virus. Avian Dis **39:**887-896.

140. **Malik, Y. S., D. P. Patnayak, and S. M. Goyal.** 2004. Detection of three avian respiratory viruses by single-tube multiplex reverse transcription-polymerase chain reaction assay. J Vet Diagn Invest **16:**244-248.

141. **Margalit, H., J. L. Spouge, J. L. Cornette, K. B. Cease, C. Delisi, and J. A. Berzofsky.** 1987. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. J Immunol **138:**2213-2229.

142. **Marien, M., A. Decostere, A. Martel, K. Chiers, R. Froyman, and H. Nauwynck.** 2005. Synergy between avian pneumovirus and Ornithobacterium rhinotracheale in turkeys. Avian Pathol **34:**204-211.

143. **Mase, M., S. Asahi, K. Imai, K. Nakamura, and S. Yamaguchi.** 1996. Detection of turkey rhinotracheitis virus from chickens with swollen head syndrome by reverse transcriptase-polymerase chain reaction (RT-PCR). J Vet Med Sci **58:**359-361.

144. **Maurer, J. J.** 2011. Rapid detection and limitations of molecular techniques. Annu Rev Food Sci Technol. **2:**259-279.

145. **McDougall, J. S., and J. K. Cook.** 1986. Turkey rhinotracheitis: preliminary investigations. Vet Rec **118:**206-207.

146. **Mebatsion, T., M. J. Schnell, J. H. Cox, S. Finke, and K. K. Conzelmann.** 1996. Highly stable expression of a foreign gene from rabies virus vectors. P Natl Acad Sci USA **93:**7310-7314.

147. **Mebatsion, T. S., M.J.; Cox, J.H.; Finke, S.; Conzelman, K.K.** 1996. Highly stable expression of a foreign gene from rabies virus vectors. Proceedings of the National Academy of Sciences of the United States of America **93:**7310-7314.

148. **Mekkes, D. R., and J. J. de Wit.** 1998. Comparison of three commercial ELISA kits for the detection of turkey rhinotracheitis virus antibodies. Avian Pathology **27:**301-305.

149. **Meyers, G., H. J. Thiel, and T. Rumenapf.** 1996. Classical Swine Fever Virus: Recovery of Infectious Viruses from cDNA Constructs and Generation of Recombinant Cytopathogenic Defective Interfering Particles. Journal of Virology **70:**1588 - 1595.

150. **Minta, Z., B. Bartnicka, and B. P.** 1995. Serological surveillance of avian pneumovirus in chicken and turkey flocks in Poland. Bulletin of the Veterinary Institute in Pulawy **39:**103-107.

151. **Mockett, A. P., D. Cavanagh, and T. D. Brown.** 1984. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41. J Gen Virol **65 (Pt 12):**2281-2286.

152. **Moore, K. M., M. W. Jackwood, and D. A. Hilt.** 1997. Identification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. Arch Virol **142:**2249-2256.

153. **Moormann, R. J. M., H. G. P. Van Gennip, G. K. W. Miedema, M. M. Hulst, and P. A. Van Rijn.** 1996. Infectious Transcribed from an Engineered Full-Length cDNA Template of the genome of a Pestivirus. Journal of Virology **70:**763 - 770.

154. **Naylor, C., K. Shaw, P. Britton, and D. Cavanagh.** 1997. Appearance of type B avian Pneumovirus in great Britain. Avian Pathol **26:**327-338.

155. **Naylor, C. J.** 1993. Studies on avian pneumoviruses including development of a vaccine. PhD thesis**:**125-130.

156. **Naylor, C. J., A. R. Al-Ankari, A. I. Al-Afaleq, J. M. Bradbury, and R. C. Jones.** 1992. Exacerbation of mycoplasma gallisepticum infection in turkeys by rhinotracheitis virus. Avian Pathology **21:**295-305.

157. **Naylor, C. J., P. Britton, and D. Cavanagh.** 1998. The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumovirus are conserved to a similar extent as those of human respiratory syncytial virus. J Gen Virol **79:**1393-1398.

158. **Naylor, C. J., P. A. Brown, N. Edworthy, R. Ling, R. C. Jones, C. E. Savage, and A. J. Easton.** 2004. Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. J Gen Virol **85:**3219-3227.

159. **Naylor, C. J., and R. C. Jones.** 1994. Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. Vaccine **12:**1225-1230.

160. **Naylor, C. J., R. Ling, N. Edworthy, C. E. Savage, and A. J. Easton.** 2007. Avian metapneumovirus SH gene end and G protein mutations influence the level of protection of live-vaccine candidates. J Gen Virol **88:**1767-1775.

161. **Naylor, C. J., C. Lupini, and P. A. Brown.** 2010. Charged amino acids in the AMPV fusion protein have more influence on induced protection than deletion of the SH or G genes. Vaccine **28:**6800-6807.

162. **Naylor, C. J., K. J. Worthington, and R. C. Jones.** 1997. Failure of maternal antibodies to protect young turkey poults against challenge with turkey rhinotracheitis virus. Avian Dis **41:**968-971.

163. **Naylor, C. J. L., C. Brown, P. A.** 2010. Charged amino acids in the AMPV fusion protein have more influence on induced protection than deletion of the SH or G genes. Vaccine **28:**6800 - 6807.

164. **Neumann, G., M. A. Whitt, and Y. Kawaoka.** 2002. A decade after generation of a negative-sense RNA virus from cloned cDNA - What have we learned? Journal of General Virology **83:**2635 - 2662.

165. **O'Loan, C. J., W. L. Curran, and M. S. McNulty.** 1992. Immuno-gold labelling of turkey rhinotracheitis virus. Zentralbl Veterinarmed B **39:**459-466.

166. **Ongor, H., M. Karahan, R. Kalin, H. Bulut, and B. Cetinkaya.** 2010. Detection of avian metapneumovirus subtypes in turkeys using RT-PCR. Vet Rec **166:**363-366.

167. **Ongor, H., M. Karahan, R. Kalin, H. Bulut, and B. Cetinkaya.** 2010. Detection of avian metapneumovirus subtypes in turkeys using RT-PCR (vol 166, pg 363, 2010). Veterinary Record **166:**397-397.

168. **Owoade, A. A., M. F. Ducatez, J. M. Hubschen, A. Sausy, H. Chen, Y. Guan, and C. P. Muller.** 2008. Avian metapneumovirus subtype A in China and subtypes A and B in Nigeria. Avian Dis **52:**502-506.

169. **Owoade, A. A. D., M. F.; Hubscen, J. M.; Sausy, A.; Chen, H.; Guan, Y.; Muller, C. P.;.** 2008. Avian Metapneumovirus subtype A in China and subtypes A and B in Nigeria. Avian Diseases **52:**502 - 506.

170. **Panigrahy, B., D. A. Senne, J. C. Pedersen, T. Gidlewski, and R. K. Edson.** 2000. Experimental and serologic observations on avian pneumovirus (APV/turkey/Colorado/97) infection in turkeys. Avian Dis **44:**17-22.

171. **Parry, J. E. S., P. V. ; Pringle, C. R. .** 1979. Pneumoviruses: the cell surface of lytically and persistently infected cells. Journal of General Virology **44:**479 - 491.

172. **Pattison, M.** 1998. Presented at the European perspective of TRT, St Cloud, Minnesota, USA, 30th June-1 July.

173. **Pedersen, J. C., D. L. Reynolds, and A. Ali.** 2000. The sensitivity and specificity of a reverse transcription-polymerase chain reaction assay for the avian pneumovirus (Colorado strain). Avian Dis **44:**681-685.

174. **Peluso, R.** 1988. Kinetic, quantitative and functional analysis of multiple forms of the vescicular stomatitis virus nucleocapsid protein in infected cells. J Virol **62:**2799 - 2807.

175. **Picault, J., P. Girauld, P. Drouin, J. Lamande, D. Toquin, C. Gueguen, V. Kles, M. Morin, M. Guittet, and G. Bennejean.** 1987. Une etiologie commune avec la rhinotracheite infectiouse de la dinde. L'Avicoulteur**:**74-75.

176. **Picault, J. P., P. Giraud, P. Drouin, M. Guittet, G. Bennejean, J. Lamande, D. Toquin, and C. Gueguen.** 1987. Isolation of a TRTV-like virus from chickens with swollen-head syndrome. Vet Rec **121:**135.

177. **Polland, B., H. M. Hafez, and L. Vasicek.** 1992. Turkey rhinotracheitis in Austria. Wiener Tierarztliche Monatsschrift **3:**34-74.

178. **Pringle, C. R.** 1998. Virus taxonomy--San Diego 1998. Arch Virol **143:**1449-1459.

179. **Qingzhong, Y., T. Barrett, T. D. Brown, J. K. Cook, P. Green, M. A. Skinner, and D. Cavanagh.** 1994. Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). Vaccine **12:**569-573.

180. **Radford, A. D., S. Dawson, K. P. Coyne, C. J. Porter, and R. M. Gaskell.** 2006. The challenge for the next generation of feline calicivirus vaccines. Vet Microbiol **117:**14-18.

181. **Randhawa, J. S., A. C. Marriott, C. R. Pringle, and A. J. Easton.** 1997. Rescue of synthetic minireplicons establishes the absence of the NS1 and NS2 genes from avian pneumovirus. J Virol **71:**9849-9854.

182. **Rautenschlein, S., Y. H. Aung, and C. Haase.** 2011. Local and systemic immune responses following infection of broiler-type chickens with avian Metapneumovirus subtypes A and B. Veterinary Immunology and Immunopathology **140:**10 - 22.

183. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. American journal of hygiene **27:**493-497.

184. **Roldao, A. O., R.; Carrondo, M.J.T.; Alves, P.M.** 2009. Error assessment in recombinant baculovirus titration: Evaluation of different methods. Journal of Virological Methods **159:**69-80.

185. **Romero, P., Z. Obradovic, and A. Dunker.** 1997. Sequence data analysis for long disordered regions prediction in the calcineurin family. Gen Inform **8:**110-124.

186. **Rubbenstroth, D., T. S. Dalgaard, S. Kothlow, H. R. Juul-Madsen, and S. Rautenschlein.** 2010. Effects of Cyclosporin A induced T-lymphocyte depletion on the course of avian Metapneumovirus (aMPV) infection in turkeys. Dev Comp Immunol **34:**518-529.

187. **Rubbenstroth, D., M. Ryll, K. P. Behr, and S. Rautenschlein.** 2009. Pathogenesis of Riemerella anatipestifer in turkeys after experimental mono-infection via respiratory routes or dual infection together with the avian metapneumovirus. Avian Pathol **38:**497-507.

188. **Sambrook, J., and D. W. Russell.** 2001. Molecular Cloning: A Laboratory Manual, 3rd ed, New York.

189. **Sato, H. Y., M. Honda, T. Kai, C.** 2011. Recombinant vaccines against the mononegaviruses - What we have learned from animal disease controls. Virus Research **162:**63 - 71.

190. **Schiricke, E.** 1984. La rhinotrachéite de la dinde: historique, évolution, symptomes et lésions moyens de lutte. L'aviculteur **442:**91 -98.

191. **Schnell, M. J., L. Buonocore, M. A. Whitt, and J. K. Rose.** 1996. The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. J Virol **70:**2318-2323.

192. **Schnell, M. J. B., L.; Whitt M.A.; Rose, J.K.** 1996. The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vescicular stomatitis virus. Journal of Virology **70:**2318-2323.

193. **Seal, B. S.** 2000. Avian pneumovirus and emergence of a new type in the United States of America. Animal Health Research Reviews**:**67-72.

194. **Seal, B. S.** 1998. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first U.S. avian pneumovirus isolate is distinct from European strains. Virus Research**:**45-52.

195. **Seal, B. S., H. S. Sellers, and R. J. Meinersmann.** 2000. Fusion protein predicted amino acid sequence of the first US avian pneumovirus isolate and lack of heterogeneity among other US isolates. Virus Res **66:**139-147.

196. **Sedova, E., D. Shcherbinin, A. I. Migunov, A. Smirnov, D. Logunov, M. M. Shmarov, L. M. Tsybalova, B. S. Naroditskiĭ, O. I. Kiselev, and A. L. Gintsburg.** 2012. Recombinant influenza vaccines. Acta Naturae **4:**17-27.

197. **Seidman, C. E., K. Struhl, J. Sheen, and T. Jessen.** 2001. Introduction of plasmid DNA into cells. Curr Protoc Mol Biol **1**.

198. **Senne, D. A., Edson.R.K., J. C. Pederson, and B. Panigrahy.** 1997. Presented at the 134th Annual Congress of American Veterinary Medical Association., Reno, Nevada, USA.

199. **Seo, S. H. W., L. Smith, R. and Collisson, E.W.** 1997. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. Journal of Virology **71:**7889-7894.

200. **Sharma, J. M., P. Chary, M. khatry, R. Cha, and J. M. Palmiquist.** 2004. Presented at the IV International Symposium on Avian Corona and Pneumovirus Infections, Rauischholzhausen, Germany, 20-24 June 2004.

201. **Shin, H. J., F. F. Jirjis, M. C. Kumar, M. K. Njenga, D. P. Shaw, S. L. Noll, K. V. Nagaraja, and D. A. Halvorson.** 2002. Neonatal avian pneumovirus infection in commercial turkeys. Avian Dis **46:**239-244.

202. **Shin, H. J., B. McComb, A. Back, D. P. Shaw, D. A. Halvorson, and K. V. Nagaraja.** 2000. Susceptibility of broiler chicks to infection by avian pneumovirus of turkey origin. Avian Dis **44:**797-802.

203. **Shin, H. J., M. K. Njenga, B. McComb, D. A. Halvorson, and K. V. Nagaraja.** 2000. Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic homology with RNA from APV isolates from domestic turkeys. J Clin Microbiol **38:**4282-4284.

204. **Starick, E., A. Romer-Oberdorfer, and O. Werner.** 2000. Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV). J Vet Med B Infect Dis Vet Public Health **47:**295-301.

205. **Stuart, J. C.** 1989. Rhinotracheitis: turkey rhinotracheitis (TRT) in Great Britain, vol. 21 Butterworth & Co Publishers Ltd., London UK.

206. **Sugiyama, Y., H. Koimaru, M. Shiba, E. Ono, T. Nagata, and T. Ito.** 2006. Drop of egg production in chickens by experimental infection with an avian metapneumovirus strain PLE8T1 derived from swollen head syndrome and the application to evaluate vaccine. J Vet Med Sci **68:**783 - 787.

207. **Taniguchi, K., and S. Komoto.** 2012. Genetics and reverse genetics of rotavirus. Curr Opin Virol. **2:**399-407.

208. **Tarpey, I., and M. B. Huggins.** 2007. Onset of immunity following in ovo delivery of avian metapneumovirus vaccines. Vet Microbiol **124:**134-139.

209. **Tarpey, I., M. B. Huggins, P. J. Davis, R. Shilleto, S. J. Orbell, and J. K. A. Cook.** 2001. Cloning, expression and immunogenicity of the avian pneumovirus (Colorado isolate) F protein. Avian Pathol **30:**471-474.

210. **Teng, M. N., and L. Collins.** 1998. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. Journal of Virology **72:**5707 - 5712.

211. **Tiwari, A., D. P. Patnayak, Y. Chander, and S. M. Goyal.** 2006. Permissibility of different cell types for the growth of avian metapneumovirus. J Virol Methods **138:**80-84.

212. **Toquin, D., M. H. Bayon-Auboyer, N. Eterradossi, and V. Jestin.** 1999. Isolation of a pneumovirus from a Muscovy duck. Vet Rec**:**680.

213. **Toquin, D., M. H. Bayon-Auboyer, D. A. Senne, and N. Eterradossi.** 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. Avian Dis **44:**977-982.

214. **Toquin, D., N. Eterradossi, and M. Guittet.** 1996. Use of a related ELISA antigen for efficient TRT serological testing following live vaccination. Vet Rec **139:**71-72.

215. **Toquin, D., O. Guiionie, C. Allee, Y. Morin, L. Le coq, F. Zwingelsteinn, V. Jestin, and N. Eterradossi.** 2006. Presented at the V. International symposium on avian corona- and pneumoviruses and complicating pathogens, RAUISCHHOLZHAUSEN, GERMANY.

216. **Toro, H., H. Hidalgo, M. Ibanez, and H. M. Hafez.** 1998. Serologic evidence of pneumovirus in Chile. Avian Dis **42:**815-817.

217. **Towsend, E., D. A. Halvorson, K. E. Nagaraja, and D. P. Shaw.** 2000. Susceptibility of avian pneumovirus isolated from Minnesota turkeys to physical and chemical agents. Avian Diseases **44:**336 - 342.

218. **Turpin, E. A., D. C. Lauer, and D. E. Swayne.** 2003. Development and evaluation of a blocking enzyme-linked immunosorbent assay for detection of avian metapneumovirus type C-specific antibodies in multiple domestic avian species. J Clin Microbiol **41:**3579-3583.

219. **Turpin, E. A., L. E. Perkins, and D. E. Swayne.** 2002. Experimental infection of turkeys with avian pneumovirus and either Newcastle disease virus or Escherichia coli. Avian Dis **46:**412-422.

220. **Turpin, E. A., D. E. Stallknecht, R. D. Slemons, L. Zsak, and D. E. Swayne.** 2008. Evidence of avian metapneumovirus subtype C infection of wild birds in Georgia, South Carolina, Arkansas and Ohio, USA. Avian Pathol **37:**343-351.

221. **Uramoto, K., E. Hakogi, T. Watanabe, Y. Ogura, M. Hataya, and K. Ohtsuki.** 1990. Primary occurring of swollen head syndrome in Japanese broiler flocks. Journal of Japanese Society on Poultry Disease **26:**247-253.

222. **Van de Zande, S., H. Nauwynck, S. De Jonghe, and M. Pensaert.** 1999. Comparative pathogenesis of a subtype A with a subtype B avian pneumovirus in turkeys. Avian Pathology **28** 239- 244.

223. **Van de Zande, S., H. Nauwynck, and M. Pensaert.** 2001. The clinical, pathological and microbiological outcome of an Escherichia coli O2:K1 infection in avian pneumovirus infected turkeys. Vet Microbiol **81:**353-365.

224. **van den Hoogen, B. G., J. C. De Jong, J. Groen, T. Kuiken, R. De Groot, R. A. Fouchier, and A. D. Osterhaus.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med **7:**719-724.

225. **Van Loock, M., T. Geens, L. De Smit, H. Nauwynck, P. Van Empel, C. Naylor, H. M. Hafez, B. M. Goddeeris, and D. Vanrompay.** 2005. Key role of Chlamydophila psittaci on Belgian turkey farms in association with other respiratory pathogens. Vet Microbiol **107:**91-101.

226. **Van Regenmortel, M. H., C. M. Fauquet, and D. H. Bishop.** 2000. Virus Taxonomy Report of the International Committee on Taxonomy of Viruses. Academic. San Diego **551:**559 - 560.

227. **Velayudhan, B. T., V. C. Lopes, S. L. Noll, D. A. Halvorson, and K. V. Nagaraja.** 2003. Avian pneumovirus and its survival in poultry litter. Avian Dis **47:**764-768.

228. **Villarreal, L. Y., P. E. Brandao, J. L. Chacon, M. S. Assayag, P. C. Maiorka, P. Raffi, A. B. Saidenberg, R. C. Jones, and A. J. Ferreira.** 2007. Orchitis in roosters with reduced fertility associated with avian infectious bronchitis virus and avian metapneumovirus infections. Avian Dis **51:**900-904.

229. **Walpita, P., and R. Flick.** 2005. Reverse Genetics of negative-stranded RNA viruses: a global prospective. FEMS Microbiology Letters **244:**9 - 18.

230. **Weber, H., T. Taniguchi, W. Müller, F. Meyer, and C. Weissmann.** 1979. Application of site-directed mutagenesis to RNA and DNA genomes. Cold Spring Harb Symp Quant Biol. **43:**669-677.

231. **Weisman, Y., C. Strengal, R. Blumenkranz, and Y. Segal.** 1988. Presented at the Thirty seventh Western Poultry Disease Conference, Sacramento, California, USA.

232. **Wertz, G. W., R. Moundy, and L. A. Ball.** 2002. Adding Genes to the RNA Genome of Vescicular Stomatitis Virus: Positional Effects on Stability of Expression. Journal of Virology **76:**7642 - 7650.

233. **Whelan, S. P. J., J. N. Barr, and G. W. Wertz.** 2004. Transcription and Replication of Nonsegmented Negative-Strand RNA Viruses. Current topics in microbiology and immunology**:**61 - 119.

234. **Wilding, G. P., C. Baxter-Jones, and M. Grant.** 1986. Ciliostatic agent found in rhinotracheitis. Vet Rec **118:**735.

235. **Williams, R. A., C. E. Savage, and R. C. Jones.** 1991. Development of a Live Attenuated Vaccine against Turkey Rhinotracheitis. Avian Pathology **20:**45-55.

236. **Williams, R. A., C. E. Savage, K. J. Worthington, and R. C. Jones.** 1991. Further studies on the development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathology **20:**585-596.

237. **Worthington, K. J., B. A. Sargent, F. G. Davelaar, and R. C. Jones.** 2003. Immunity to avian pneumovirus infection in turkeys following in ovo vaccination with an attenuated vaccine. Vaccine **21:**1355-1362.

238. **Worthington, K. J. C., R.J. Jones, R.C.** 2008. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. Avian Pathology **37:**247-257.

239. **Wyeth, P.** 1990. Turkey rhinotracheitis and swollen head syndrome cause heavy loss. Poultry Digest**:**16 - 21.

240. **Wyeth, P. J., R. E. Gough, N. Chettle, and R. Eddy.** 1986. Preliminary observations on a virus associated with turkey rhinotracheitis. Vet Rec **119:**139.

241. **Yu, D., Z. Han, J. Xu, Y. Shao, H. Li, X. Kong, and S. Liu.** 2010. A novel B-cell epitope of avian infectious bronchitis virus N protein. Viral Immunol **23:**189-199.

242. **Yu, L. L., W. Schnitzlein, W.M. Tripathy, D.N. and Kwang, J.** 2001. Study of protection by recombinant fowl poxvirus expressing C-terminal nucleocapsid protein of infectious bronchitis virus against challenge. Avian Diseases **45:**340-348.

243. **Yu, Q., C. N. Estevez, J. P. Roth, H. Hu, and L. Zsak.** 2011. Deletion of the M2-2 gene from avian metapneumovirus subgroup C impairs virus replication and immunogenicity in Turkeys. Virus Genes **42:**339 - 346.

ACKNOWLEDGMENTS

I would like to thank all the wonderful people I have met and worked with during these years and who gave me their support and help. In particular:

Clive J. Naylor

Professor R. Jones

Elena Catelli

Caterina Lupini

Mattia Cecchinato

Paul Brown

Jayne Clubbe

Professor J. Bradbury

Kannan Ganapathy

Cynthia Dare

Ann Forrester

Carol Savage

Linda Greatwitch

Christine Yavari

Enrico Ricchizzi

Camilla Brena

Andrea, Maria e Valeria

My Family