Avian metapneumoviruses expressing Infectious Bronchitis virus genes are stable and induce protection

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ABSTRACT

The study investigates the ability of subtype A Avian metapneumovirus (AMPV) to accept foreign genes and be used as a vector for delivery of Infectious bronchitis virus (IBV) QX genes to chickens. Initially the GFP gene was added to AMPV at all gene junctions in conjunction with the development of casseted 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 furthermore, 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 major recombinant tracheal replication, were largely absent.

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1. Introduction

Avian metapneumovirus (AMPV) is a major endemic respiratory pathogen of global domestic poultry [1], and secondary infections can exacerbate the disease [2]. AMPV belongs to the subfamily Pneumovirinae, genus Metapneumovirus [3] of which four subtypes have been recognized (A, B, C and D) [4–6]. The genome has 8 genes, with genome sizes typically close to 13.5 kb [7]. Reverse genetics (RG) systems for subtypes A and C have allowed their rational mutation [8,9]. To date RG has produced viruses with deletions, gene modifications and reporter gene insertions [8,10–12]. Some studies have considered the replicative ability of recombinants in vivo [11,12] but not foreign viral gene insertion, nor the genetic stability of expanded genomes.

Avian infectious bronchitis (IBV) is the major worldwide endemic respiratory pathogen of chickens (family coronavirus, genus gammacoronavirus) which, like AMPV primarily infects the respiratory tract but can also infect the kidneys, intestine and reproductive system [13]. IBV epidemiology is characterized by the emergence of virulent variants which evade prevailing IBV immunity. In the last decade the QX genotype [14] has emerged, which predominantly affects the respiratory tract and kidneys [15]. It was first recognised in China and has continued to spread to much of Europe and Asia. In this study, novel QX candidate vaccines based on AMPV vectors were constructed and tested.

The major IBV surface protein spike S0 becomes cleaved into the outer, antigenically important S1, and inner membrane bound, S2 section [13]. Previously inoculation of S1 expressed in baculovirus was shown to induce protection [16]. The internal nucleocapsid (N) protein can also induce protective immunity [17,18] involving both T and B lymphocyte epitopes [18,19]. Here two different QX S1 genes and a QX N gene were inserted into AMPV, both to assess the vector's acceptance of foreign genes, and its suitability for delivery of IBV genes to chickens.

Three AMPV genomes acted as vectors. Prior to introduction of S1 or N genes, a cloning site flanked by a transcription start and stop sequence, was added to intergenic regions of full length (FL) DNA genome copies. To aid IBV recombinant optimization, green fluorescent protein (GFP) was initially added to these sites and recombinant viruses recovered; then AMPV S1 and N recombinant viruses were constructed. The two S1 genes used differed by a 15 nucleotide deletion present in a proportion of the donor virus.

Recombinant viruses were tested in chickens in two experiments to determine their protective capacity. IBV inoculation of chickens leads to infection of and damage to the tracheal ciliated epithelium [20]. Loss of cilial motility is readily observed [21] and protection is considered best assessed by the maintenance of motility following challenge [22], as also adopted in European Pharmacopoeia, IBV vaccine monographs. To assess replication of recombinants, real time RT PCR was performed on material from choanal swabs while induced specific antibody to IBV and AMPV was measured by ELISA and haemaglutination inhibition (HI).

2. Materials and methods

2.1. Addition of GFP to AMPV

Three subtype A AMPV viruses were employed as vectors for GFP and later IBV genes. First was a German field isolate (Virus A) passaged in Vero cells [9] and found avirulent in turkeys [12], second was Virus AvF which contained an F gene modification found to better induce protection in turkeys [12] and third was 309/04, a virulent field isolate deriving from a subtype A vaccine and arising in field conditions [23].

Seven versions of virus A [12] FL cDNA were made by site directed mutagenesis, as outlined in Fig. 1. An Xho1 restriction endonuclease (RE) site was added to each intergenic region, following the preceding gene stop signal and prior to the downstream gene start signal. A cloning cassette flanked by cut Sal 1 RE sites, allowing gene insertion and transcription, was ligated into each cut Xho1 RE site of the seven FLs. After cloning into STB12 cells (Invitrogen, according to manufacturer's recommendation (AMR)), 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 1) and these were added to the seven FL cDNAs by ligation after Xho1 digestion. Colonies containing DNA of correct orientation and sequence were selected by sequencing. FLs containing GFP were recovered by RG [9]. GFP expression was assessed by fluorescence microscopy. Virus viabilities were evaluated as maximum titres obtained following three Vero passages.

2.2. 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 °C. As the genome sequence was unknown, a range of IBV genomes were aligned to identify conserved regions within, and flanking, S1 and N genes. These were used for the design of RT-PCR and sequencing primers, as detailed in Table 1. Sequencing of the S1 gene amplicon revealed two populations, one of which showed a 15 nucleotide deletion (del) (5′-UGUUUGAUUCUGAUA-3′) between bases 59 and 73.

2.3. Predictive computational comparison of S1 populations

S1 genes with and without the deletion were compared. 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 prediction used the VL-XT predictor on the PONDR server [24]. Prediction of immunodominant helper T-lymphocyte antigenic sites from primary sequence data was by analysis of the occurrence of amphipathic fragments using the AMPHI algorithm [25]. The predictive algorithms had been previously shown correct in more than 75% of cases.
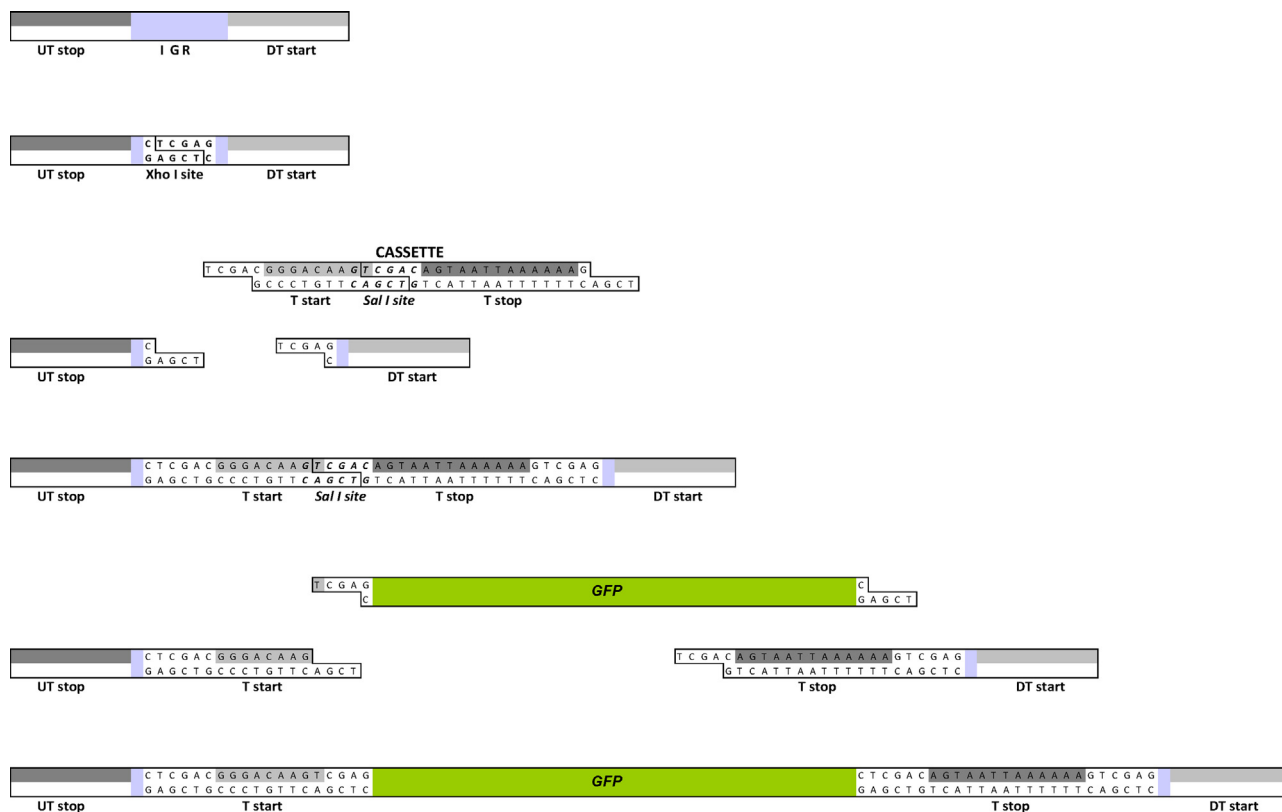

Fig. 1. Schematic representation of the strategy used for adding genes to cloned AMPV genome copies. An Xho1 RE site, then standardized cassette and finally chosen gene for expression (illustrated by GFP) were added to intergenic regions (IGR) between upstream gene transcriptional stops (UT stop) and the downstream gene transcriptional starts (DT start).

Table 1
Oligonucleotide primer sequences used.

	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

2.4. IBV S1 and N amplification for insertion

For gene insertion, Xho I RE sites were added to S1 and N gene extremities by RT-PCR using modifying primers S1 start xho+ S1 stop xho neg, N start xho+ and N stop xho neg (Table 1).

2.5. IBV gene insertion into AMPV cDNAs

IBV genes were inserted into FLs A, AvF [12] and FL 309/04 [23] with the cassette at the MF intergenic region. 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 2.

2.6. Recovery of virus

Recombinant viruses were rescued from FLs [9] then 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 titres were calculated [26]. Virus stocks of 1.5 ml aliquots were stored at −80 °C.

Table 2
Details of the constructed recombinant cloned FL cDNAs.

Clone name	Source virus	IBV QX insert	Intergenic region[e]
$A_{del\ S1\ MF}$	A[a]	Deleted S1[d]	MF
$A_{full\ S1\ MF}$	A	Full S1	MF
$AvF_{del\ S1\ MF}$	AvF[b]	Deleted S1[d]	MF
$AvF_{full\ S1\ MF}$	AvF	Full S1	MF
$AvF_{N\ MF}$	AvF	N	MF
$AvF_{full\ S1\ MF + N\ GL}$	AvF	Full S1 + N	MF + GL
$309_{full\ S1\ MF}$	309/04[c]	Full S1	MF

[a] Subtype A virus isolated in Germany.
[b] Virus A with F gene modifications increasing induced protection in turkeys.
[c] Field virus deriving from an subtype A type registered vaccine.
[d] IBV QX S1 containing a 15 base deletion between nucleotides 59 to 73.
[e] Location in cloned AMPV of the inserted gene(s).

2.7. S1 and N gene messenger RNA RT-PCRs

To verify the transcription of inserted S1 and N genes in Vero cell grown virus, a previously reported protocol [10] was used, except that primers within those genes were S1 1380+ and N 920+ for S1 and N genes respectively (Table 1).

2.8. Expression of inserted genes

S1 and N protein expression were assessed using immunofluorescence (IF) on AMPV recombinant infected Vero monolayers, using a polyclonal chicken antiserum (GD) anti QX for S1 expression and a monoclonal mouse antibody (Biozol) for N gene expression. Appropriate FITC conjugated antibodies were used to visualize specific S1/N proteins (AMR).

2.9. Chicken experiment 1

Approximately 70 one-day-old SPF chickens were divided into seven groups, each containing ten animals. In groups one to four, birds were inoculated by eyedrop with 4 $\log_{10}$ TCID50 $A_{full\ S1\ MF}$, $AvF_{full\ S1\ MF}$, $A_{del\ S1\ MF}$ and $AvF_{del\ S1\ MF}$ respectively. The AMPV control group was inoculated with AvF. At 21 days post vaccination (dpv), all birds, and half the unvaccinated control (C+) were challenged with 4 $\log_{10}$ TCID50 QX IBV strain by eyedrop, while the other controls remained unvaccinated (C−). For sampling, of tracheas and kidneys, half the chickens per group were humanely killed at 4 days post challenge (dpc) and the remainder at 6 dpc.

2.10. Chicken experiment 2

Approximately 60 one-day-old SPF chickens were divided into six groups of ten birds, of which four were inoculated with 4 $\log_{10}$ TCID50 of viruses $309_{full\ S1\ MF}$, $AvF_{full\ S1\ MF + N\ GL}$, $AvF_{N\ MF}$ and $AvF_{full\ S1\ MF}$ virus by eyedrop. The remaining groups acted as controls. At 21 dpv, all vaccinated birds and half the unvaccinated controls (C+) were challenged with 4 $\log_{10}$ TCID50 of IBV QX by eyedrop, while the other controls remained unvaccinated (C−). In each group, five birds were humanely killed at 4 dpc and five at 6 dpc and tracheas and kidneys were collected.
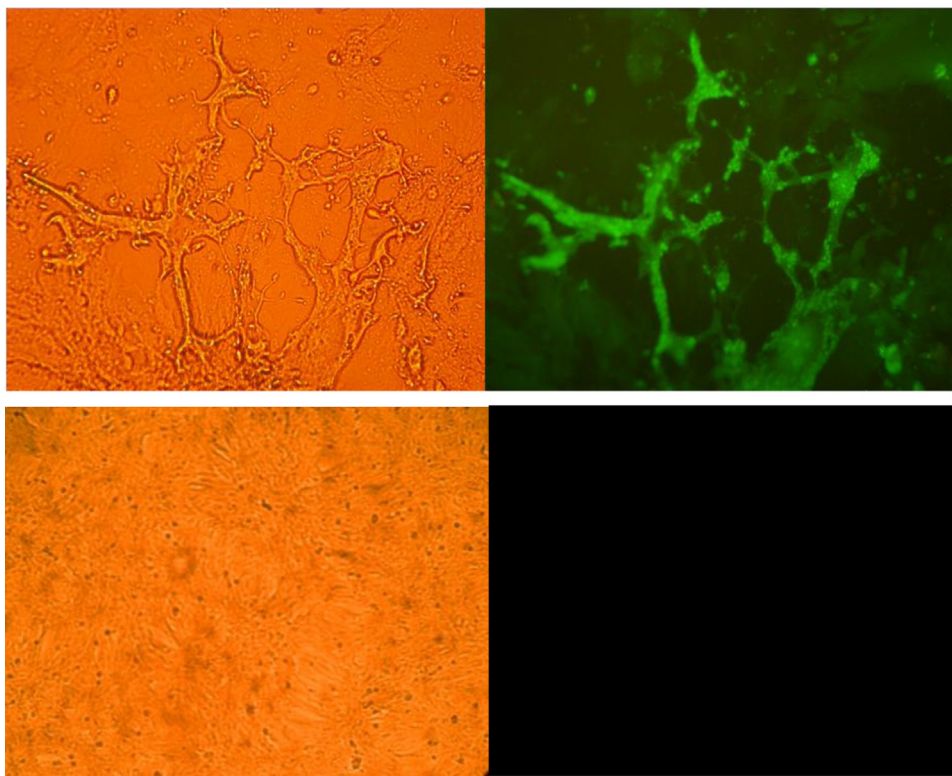
Fig. 2. TOP Vero cell monolayer infected with virus expressing GFP between MF viewed under white (left) and ultraviolet illumination (right) BELOW Uninfected Vero cell monolayer under white (left) and ultraviolet illumination (right).

2.11. Serology

Chickens from each group were bled at 18 dpv for IBV and AMPV serology. Sera from birds receiving S1 recombinant AMPVs were tested using an IBV QX HI test, while those receiving either recombinant containing the N gene were tested by IBV HI and ELISA (Biochek, AMR). AMPV ELISA (IDEXX, AMR).

2.12. 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 post vaccination (dpv) for AMPV real time RT-PCR [27] to assess recombinant replication.

2.13. 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. Individual sections were classified as either containing beating cilia or beating being entirely absent.

2.14. Statistical analysis

Statistical analysis of cilial motility was performed using the Chi-square test. A p value < 0.05 was considered statistically significant.

2.15. Clinical signs

Birds were examined daily for the presence of nasal exudate and generally for overall health.

3. Results

3.1. Recombinant AMPV GFP construction

The GFP gene was added to modified FL AMPV copies and viruses were recovered. Maximum $TCID_{50}$ titres per ml of Vero cell lysate, following three Vero cells passages, were low when the inserted gene was positioned at a gene junction close to the virus leader. Hence with GFP placed between N and P genes, the titre was 2.1 while in all other positions it exceeded 4.0. At the MF position it was 5.0. For all constructs, strong fluorescence was observed when infected Vero cells were viewed by UV microscopy for all constructs as illustrated for recombinant GFP MF (Fig. 2).

3.2. Computational analysis

Protean suite software analysis predicted S1 proteins to share the same physicochemical properties and PONDR predicted no differences to the disordered region. The Protean suite predicted the proportion of alpha helix to remain constant while a difference of beta pleated sheets was evident. 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 acids 264-268 and 383-388).

3.3. IBV recombinant AMPV construction

Virus was rescued from AMPV FL cDNAs containing IBV genes as confirmed by cytopathic effect typical of AMPV on Vero cell monolayers. Viruses were sequenced to after three passages. RT-PCR of virus mRNAs confirmed transcription of the inserted IBV genes (data not shown). Expression of IBV proteins was confirmed in all recombinant viruses by IF (Fig. 3).
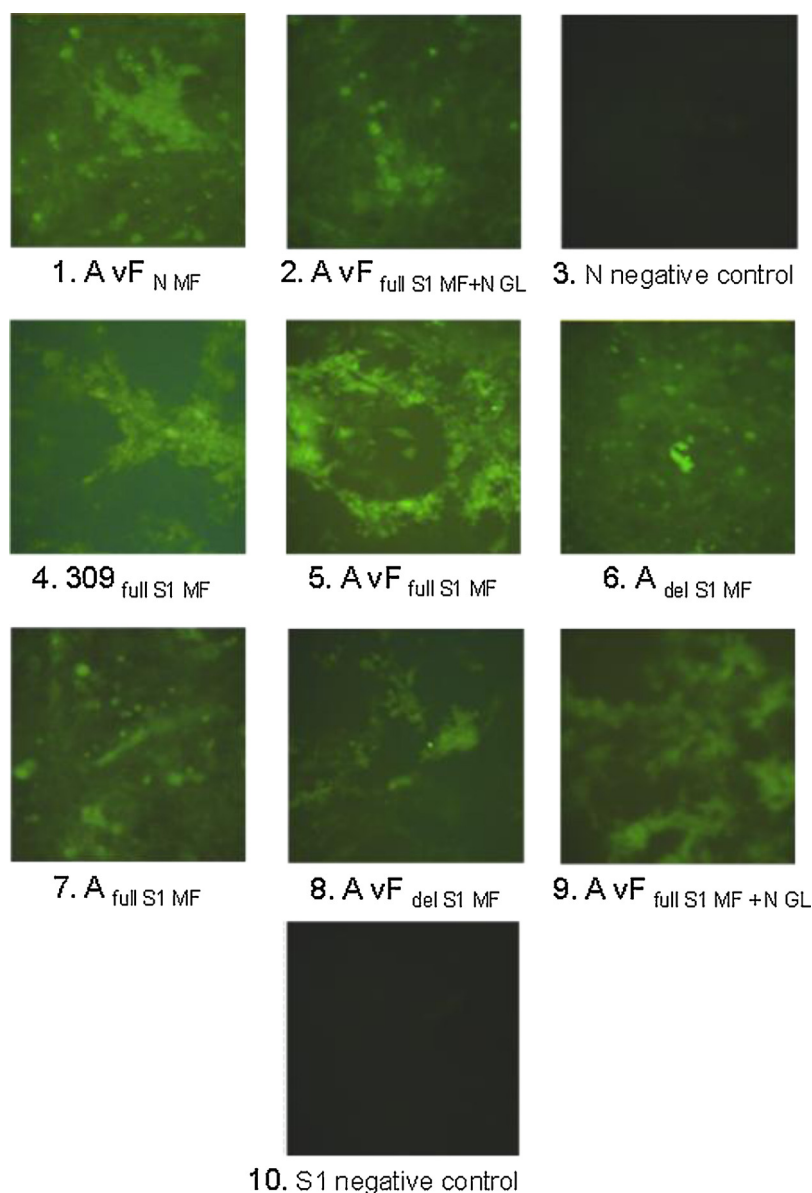
Fig. 3. Vero cell monolayers infected with AMPV recombinant viruses viewed by UV microscopy. Viruses 1 to 3 used IBV N monoclonal serum and 4 to 10 used IBV polyclonal chicken serum.

3.4. Serology

IBV and AMPV antibody responses were not generally detectable (Table 3). For IBV in experiment 1, a single bird in group $AvF_{del\ S1\ MF}$ and two birds in group $AvF_{full\ S1\ MF}$ had detectably seroconverted by HI. For AMPV ELISA serology, two seroconversions were detected in the AvF control group (experiment 1) and three in the $309_{full\ S1\ MF}$ group (experiment 2).

3.5. AMPV real time RT-PCR

Except for $309_{full\ S1\ MF}$, real time RT-PCR showed minimal AMPV recombinant replication (Table 3).

3.6. Tracheal motility following challenge

At 4 dpc, sections from all birds challenged with IBV were ciliostatic. At 6 dpc, some sections from birds previously inoculated with AMPV QX recombinants showed cilial motility, with those birds given $AvF_{full\ S1\ MF+N\ GL}$ showing most, followed by $AvF_{N\ MF}$ and with the least for AvF viruses expressing only the S1 gene. In all cases the distribution of beating rings within any group was even. No differences were observed between recombinant AMPVs expressing the full and deleted S1. Comparing AMPV vectors with identically positioned inserts, A led to greater cilial recovery than either 309/04 or AvF. See Table 3 for details.

3.7. Statistical analysis

Significantly increased cilial activity, calculated by comparing each group to the respective positive control, was found for birds vaccinated with $A_{full\ S1\ MF}$ ($p = 0.0002$), $A_{del\ S1\ MF}$ ($p = 0.0008$), $AvF_{full\ S1\ +N\ GL}$ ($p = 0.00001$) and $AvF_{N\ MF}$ ($p = 0.0002$) and analysis between these groups did not reveal any significant differences.

Table 3
Chickens experiments 1 and 2: post vaccination virus detection by real time RT-PCR, pre challenge serology and cilial activity post challenge.

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

50 rings prepared from 5 birds from each group, each sampling day.

[a] Days post vaccination.
[b] Days post challenge.
[c] Not done.

3.8. Clinical signs and gross lesions

No signs were detected after vaccination. In experiment 1, clinical signs were observed in three birds at 6 dpc. One positive control bird and two AvF control birds were humanly killed after displaying lethargy, ruffled feathers and reduced response to external stimuli. Gross kidney lesions, typical of QX IBV infection, were detected at post-mortem examination in the same birds. No signs or lesions were seen in experiment 2.

4. Discussion

When IBV MF recombinants were used to inoculate one-day-old chickens, many induced IBV protection of the trachea, yet serology and real time RT PCR virus detection indicated poor tracheal replication. Surprisingly, recombinants replicating least induced most protection whereby recombinants based on virus A protected best while virus 309/04 induced no detectable protection. Despite computer analysis predicting T cell epitopes differences concerning the S1 15 nucleotide deletion, no protection differences were observed.

The primary site of AMPV and IBV replication in chickens is the upper respiratory tract, hence an AMPV based IBV recombinant might be predicted to be ideal for inducing IBV protection. Conventional wisdom suggests that the minimal levels of upper respiratory tract replication detected in our study, led to the observed protection. Therefore any AMPV recombinant replicating better might yield greater protection. Growing worldwide commercial field evidence is indicating that subtype B AMPV better infects commercial chickens than subtype A. If a subtype B RG system becomes available, protection afforded by any resultant IBV recombinants will be keenly compared to the current study, which counter-intuitively suggests that recombinant AMPVs inducing the best protection replicate least well. If more protection inducing recombinants are found not to appreciably replicate in the respiratory tract or produce generalised seroconversion, it will become necessary to investigate whether replication might occur at another site or alternatively protection may result from a yet unrecognized mechanism. There is already some evidence that, AMPV replication in the respiratory tract of turkeys does not imply induction of AMPV protection [12], protection following live vaccination does not require initial replication in the trachea [28] and AMPV vaccination can lead to apparent protection without AMPV seroconversion [29,30].

For the first time, AMPV recombinants are reported carrying foreign viral genes. Previously AMPVs were shown highly stable both in cell culture and during natural passage [20] as has also been reported for other members of the Mononegavirales such as vesicular stomatitis and rabies viruses [31,32]. This contrasts with single strand positive viruses such as IBV and feline calicivirus where minimal passage readily results in consensus sequence mutations [13,33]. Furthermore, the recombinant AMPV genome carrying more than 3000 extra nucleotides (IBV S1 and N genes) was stable with respect to functionally irrelevant inserted genes, and their presence did not appreciably reduce virus viability, if distanced beyond the phosphoprotein gene.

Our study suggests that a range of AMPV vectored candidate vaccines could be readily prepared. For IBV, this is in marked contrast to conventional attenuation where more than 100 egg passages is typically required. Such AMPV recombinants would have greater genetic stability in two key respects. Firstly instability on simple passage implies that any live vaccine will be genetically heterogeneous. Of greater significance is the ability of IBV live vaccines to recombine with other IBV genotypes during co-infection, via homologous recombination, thus potentially leading to new field genotypes. In contrast, AMPV has been shown to be stable with respect to multiple passage while the absence (or extremely low frequency) of homologous recombination avoids recombinant generation.

The nature of the protective immune response to IBV is not well understood [13]. While virus AvF containing N gene alone or N + S1 genes together protected tracheas better than AvF containing the S1 gene alone, the expression of the inserted genes would need to be quantified, before conclusions about the relative contribution of each gene to protection, could be drawn. Previous studies indicate that the internal N protein is able to stimulate a cell mediated immune response (CMI) [18] whereas the exposed S1 protein would be more likely to stimulate antibody production [34–36], hence our study adds weight to the notion that both a CMI and antibody response are important in protection.

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. Those recombinant AMPV-IBV viruses are genetically stable and able to incorporate inserted genes totalling at least 3000 nucleotides; though the upper limit is yet to be established. It is likely that further developments will lead to flexible vaccines of greater efficacy as well as providing a better

understanding of the essential components for induction of effective protection against IBV as well as other avian viruses.

Conflict of interest

There is no conflict of interest.

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