MOLECULAR STUDIES TOWARDS IMPROVED AVIAN METAPNEUMOVIRUS VACCINES

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Contents

Preface

The experimental work carried out in this thesis was performed at the University of Liverpool in the Institute of Infection and Global Health, Department of Veterinary Pathology, between 2008 and 2011 and was supported by Fort Dodge Animal Health, Lohmann Animal Health and RCUK Fellowship EP/E50065X/1.

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Abstract

The work undertaken in this thesis centres around molecular studies on avian metapneumovirus (AMPV) subtypes A and B. The findings and outcomes may be of use in the future development of improved vaccines against the disease.

Since the emergence of AMPV a range of live attenuated vaccines have been developed and these are used in the control of the disease throughout the world. However disease outbreaks still occur and improved vaccines are needed for the control, or better still the eradication of AMPV. Commercially, vaccines have been marketed with the belief that they are more efficacious in the species from which the progenitor virus was derived. To investigate this at a molecular level, the first part of the study compared full length (FL) genome sequences of chicken and turkey-derived AMPVs to identify potential species specific regions of sequence. A FL RT-PCR and sequencing system was developed for AMPV subtype B, and used along with an already established system for subtype A to determine the FL sequences from a range of field strains and commercially available vaccines. The nucleotide (nt) sequences and predicted amino acid (aa) sequences were aligned and compared. In parallel, turkey-derived viruses were multiply passaged in chicken tracheal organ culture (TOC)to investigate whether viruses would evolve towards potential species specific motifs. A conclusive comparison study for AMPV subtype A could not be completed due to problems sourcing subtype A chicken-derived viruses. For AMPV subtype B numerous sequence differences between viruses were identified, however none were specific for the host species.

The second part of this study investigated one of the two major subtype A commercially available live vaccines, both derived from a highly virulent UK field strain, #8544. Previously, improvements of those vaccines, by use of reverse

genetics technology (RG), was found to be hampered by the inability of #8544 to replicate in the commonly exploited Vero cell based RG system. In the present study a FL DNA copy of one of the Vero grown, #8544 derived, live vaccines was modified, using the RG system, to create an exact FL DNA copy of #8544. This vaccine sequence was then re-introduced systematically, leading to the identification of a single coding substitution in the M2:2 reading frame responsible for replication and virus recovery in Vero cells. Ablation of M2:2 also resulted in replication and virus recovery in Vero cells. M2:2 sequence analysis of seven AMPVs found Vero cell adaption to be associated with non similar amino acid changes in M2:2. The M2:2 modification of field virus #8544 will enable research leading to improved vaccines.

RG systems are now commonly being employed to aid the development of improved vaccines. However, as yet a system does not exist for AMPV subtype B. In the final part of this study the FL genome sequence of AMPV subtype B was successfully cloned. This is a major step in the development of a functional AMPV subtype B RG system.

Acronyms and abbreviations

aa	amino acid
AMPV	avian metapneumovirus
bp	base pairs
BRSV	bovine respiratory syncytial virus
^{o}C	degree Celsius
cDNA	complimentary deoxyribonucleic acid
CEF	chick embryo fibroblasts
CEL	chick embryo liver
CER	chick embryo related
CPE	cytopathic effect
DNA	deoxyribonucleic acid
EM	electron microscopy
ELISA	enzyme-linked immunosorbent assay
F	fusion protein
FL	full length
G	attachment protein
g	gram
hMPV	human metapneumovirus
HRSV	human respiratory syncytial virus
IBV	infectious bronchitis virus
IP	immunoperoxidase
IF	immunofluorescence
kb	kilobases
kbp	kilobase pairs
L	polymerase protein
M	matrix protein
Mg	Mycoplasma gallisepticum
µg/ml	microgram/millilitre
mg	milligram
µl	microlitre
ml	millilitre
mM	milli-Molar concentration
mRNA	messenger ribonucleic acid
MW	molecular weight

N	nucleocapsid protein
ng/µl	nanogram/microlitre
nm	nanometre
nt	nucleotides
ORF	open reading frame
P	phosphoprotein
PCR	polymerase chain reaction
pi	post infection
RE	restriction endonuclease
RG	reverse genetics
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
SDM	site-directed mutagenesis
SH	small hydrophobic protein
SHS	swollen head syndrome
SPF	specific pathogen free
T7	T7 polymerase
Taq	Thermus aquaticus
TOC	tracheal organ culture
TRT	turkey rhinotracheitis
U/µl	units/microlitre
UV	ultraviolet
VN	virus neutralisation

Chapter 1

Introduction and aims

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Chapter 1

Introduction and aims

1.1 Introduction

Avian metapneumovirus (AMPV) is a respiratory pathogen which causes significant economic losses to the poultry industry. The virus causes acute respiratory tract infection in turkeys characterized by sneezing, tracheal râles, swollen sinuses and nasal discharge (166). Infection of chickens results in a drop in egg production from laying birds and can be associated with swollen head syndrome (SHS) (57). At present, there are four known subtypes A, B, C and D. Subtypes A and B have now been reported in most countries worldwide, whilst subtype C has only been reported in the USA (196), France (217), China (208, 230) and in one case in Korea (138). Subtype D has only been reported in France (16).

Since the virus first emerged in South Africa in 1978 (32), a range of live attenuated vaccines have been developed to control the disease. Commercially, species specific vaccines have been marketed with the belief that they are more efficacious when used in the species from which the progenitor field virus was derived. In support of this, studies have indicated contrasting outcomes following experimental infection with chicken or turkey-derived AMPV field isolates in terms of pathogenesis and susceptibility (33, 34, 63, 94). Experimental infections in chickens and turkeys led to clinical signs that were reported to be more marked when infecting birds with virus originating from the same species (63). In contrast a recent study reported that a chicken isolate gave more clinical signs in turkeys than in chickens (136).

This lack of a full reproducible chicken challenge model means such clinical differences can be difficult to reproduce. In addition, varying factors such as virus

virulence, virus subtype, inoculation routes, dosage, age, gender and immune system differences in the birds may also play a role in varying outcomes (8, 64, 189).

Whilst current commercial vaccines have kept AMPV largely under control reports of disease in vaccinated flocks still occur (10, 11, 39, 42, 48). Poor vaccine application is suggested to be amongst one of the reasons for cases of disease in the field (39). Due to its instability, passage of vaccine-derived virus through unvaccinated birds within a flock can lead to a reversion to virulence, following a very small number of mutations, and avoidance of vaccine induced immunity (36, 44, 149, 190). However, under experimental conditions disease was observed following challenge despite the careful application of vaccine (39). Recently isolated field virus strains have been shown to cause disease and to avoid immunity provided by the current vaccines. Nucleotide changes in the small hydrophobic (SH) and / or attachment (G) genes, compared to the vaccine strain and earlier isolates, are believed to play a major role in virus evolution and resulting disease in vaccinated flocks (10, 39). Many studies have described disease caused by an AMPV with a subtype different to that of the vaccine strain applied (10, 11, 48, 149, 161, 221). A study by Van de Zande et al (2000) found heterologous protection declined quickly following vaccination, whilst homologous protection was maintained (222). These findings were supported by Ricchizzi et al (2009) who described the failure of a subtype B vaccine to protect against a subtype A vaccine derivative, six weeks after its administration (190). Stable vaccines, which are also relevant to the prevailing circulating field strains, are needed for the complete control of AMPV in the future.

Current live subtype A commercial vaccines have been developed following the adaptation of a virulent field strain to cell culture, with subsequent virus attenuating passages (234). This adaptation and attenuation process leads to mutations which are likely to be involved in either the adaptation to the cell culture system, or in the

reduction of virulence. Very few mutations are needed to attenuate a virus and confer protection against challenge, but likewise, very few are needed to re-establish virulence. More recently, AMPV subtype A reverse genetics (RG) systems have been employed to aid the development of future recombinant vaccines with increased stability. A DNA copy of a field virus genome can be manipulated to study effects of whole or partial gene deletions, or single point mutations, on virus pathogenesis or virulence (29, 146, 164, 168). However, it is essential that candidate vaccine field virus to be studied in this approach can replicate in the cell culture utilized by the RG system.

1.2 Aims

The initial aim of this study was to determine if species specific sequences exist between chicken and turkey-derived AMPVs. Identification of conserved motifs relating to either chicken or turkey-derived virus sequences may support the need for homologous vaccines. A molecular comparison of full length (FL) subtype A and B chicken and turkey derived isolates was made including nucleotide (nt), predicted amino acid (aa) and non-coding intergenic sequences. In parallel, turkey-derived viruses were multiply passaged in chicken tracheal organ cultures (TOC) and re-sequenced to assess potential evolution of species specific motifs.

The second part of this study focused on a highly virulent AMPV field strain # 8544 and its Vero cell adapted derivative, which is one of the major commercial subtype A live vaccines. Previously, during the development of the vaccine at Liverpool, virus #8544 had been attenuated in Vero cells following a previous lengthy adaptation to chick embryo liver cells (CEL). This attenuated vaccine strain grew readily in Vero cell culture, and a FL DNA copy of the vaccine and derivatives could be recovered in the Vero cell based RG system (29, 234). Here, a FL DNA copy of field strain #8544 was developed to enable the generation of future vaccine candidates, with improved

stability, closely related to this particular strain. However it could not be recovered in the Vero cell based RG system. Sequencing revealed nine nt substitutions had occurred during the generation of the vaccine. We investigate which of these substitutions allowed growth in Vero cells and the rescue of a FL DNA copy of the vaccine. Vaccine nt mutations were introduced to a FL DNA copy of field strain #8544 in a stepwise fashion. A single nt mutation within the M2:2 reading frame, responsible for a non conservative aa substitution, was found to facilitate growth. To determine whether the base mutation had modified the M2:2 gene to one more suitable for Vero growth, or growth became possible because a functional M2:2 was no longer present, #8544 derived clones with ablated M2:2 start codons were developed.

Finally, a FL subtype B AMPV clone was developed to enable future studies into this subtype and the generation of improved recombinant vaccines using a RG system. The study made use of previously cloned sections of subtype B AMPV sequence and a subtype B vaccine virus to generate a complete cDNA clone.

Chapter 2

Literature review

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Chapter 2

Literature Review

2.1 Introduction

AMPV is a respiratory pathogen primarily affecting turkeys and chickens worldwide. The disease, initially named turkey rhinotracheitis (TRT), was first detected in 1978 in South Africa (32) where it spread rapidly through commercial turkey flocks, destroying the industry. In the mid 1980s the disease was reported in the UK (6, 157, 233, 238) and France (91).

In turkeys, the virus causes acute respiratory tract infection and in breeders egg production and quality is often affected (205). Its role as a primary pathogen in chickens is less defined. It is often linked with SHS which appeared in the UK around the same time (170). Clinical signs have been reported to be milder than those seen in turkeys, and it is not always associated with disease (65).

There are four known AMPV subtypes, A, B, C and D. Subtypes A and B are the most prevalent and have been reported in Western Europe and many other countries around the world. Subtype C emerged in 1996 after an outbreak at a commercial turkey farm in the state of Colorado, USA (198). Although it was successfully eradicated from Colorado, it spread to Minnesota and was responsible for serious economic losses to the industry. It has since been detected in a range of wild birds in the USA (220), and in other avian species in France (217), China (208) and Korea (138). Subtype D, isolated from infected turkeys in France, was shown to be genetically different to A, B and C based on sequence analysis of the fusion (F), attachment (G) and polymerase (L) genes (16). More recently the FL sequence of this virus was compared with all the other subtypes. A closer relationship with

subtypes A and B than with subtype C was evident throughout the majority of the genome (28).

2.2 The Virus

2.2.1 Taxonomy

AMPV is a member of the family Paramyxoviridae, subfamily Pneumovirinae. It was initially placed in the genus Pneumovirus after morphological, mRNA and polypeptide comparisons revealed it shared similar features with human and bovine respiratory syncitial viruses (HRSV and BRSV), and Pneumovirus of mice (PVM) (40, 52, 145, 238). It has since been re-classified as the type species of the newly created genus Metapneumovirus after it was found to lack two non structural proteins, NS1 and NS2, and to have a different genome organisation following the matrix (M) gene when compared to other Pneumoviruses (137, 144, 183, 186, 241). It was the sole member of this genus until 2001, when what was believed to be a new virus was found to be the cause of respiratory illness in young children in the Netherlands. Based on sequence homology and gene order it was placed in the genus alongside AMPV and named human metapneumovirus (hMPV).

2.2.2 Morphology

AMPV, visualised by electron microscopy (EM), have highly pleomorphic enveloped virions fringed with closely spaced surface projections 13 to 15nm in length. Observations include spherical forms, reported to have a diameter of 80-200nm, and filamentous forms with varying lengths, some in excess of 1000nm. The helical nucleocapsid, containing the viral genome and key proteins, has a diameter of 14nm and a pitch of 7nm. These measurements closely agree with other viruses in the genus Pneumovirus, however the nucleocapsid structure of HRSV and BRSV is

more rigid than that of AMPV. Pneumovirus nucleocapsids are smaller than those of other Paramyxoviruses (32-34, 52, 79, 92, 125, 157, 238).

2.2.3 Physiochemical properties

AMPV, in agreement with other members of Pneumovirinae is not associated with hemagglutination or neuraminidase activity (92, 238). Early studies reported the virus to be stable between pH 3.0 and 9.0, sensitive to a range of detergents and lipid solvents, and inactivated after 30 minutes at 56^{o}C. In sucrose gradients it has a buoyant density of 1.21 g/ml (34, 52, 54). More recently, subtype C AMPV was shown to be viable after 26 weeks following storage at -70 ^{o}C and -20 ^{o}C, and to survive for less than 6 hours at 50^{o}C. Virus titre was unaffected after 1 hour at pH varying between 5.0 and 9.0 (219).

2.2.4 Nucleic acids

AMPV has a non-segmented, single-stranded, negative-sense RNA genome (184) which has been shown to differ slightly in length between subtypes A, B, C and D.

Sequencing of subtype A genes (140, 143, 144, 188, 239-241) combined with the leader and trailer sequence, and the confirmation that NS1 and NS2 genes are absent, has revealed a total genome length of 13,373 nt (186). All genes of subtype B have been sequenced with a total reported length of 13,508 nt (115, 129, 140, 163, 187, 206). Between subtypes A and B the nt sequence identity is 56-61% whereas within a subtype it is 97-99% (150).

Sequencing of all subtype C genes has been completed (5, 72, 73, 100, 101, 150, 196, 215, 243), however early findings regarding the final length proved contradictory. Govindarajan et al (2005) reported a length of 14,150nt (99) whereas

Lwamba et al (2005) suggested a shorter length of 13,134nt (150). The variations were a result of different sized G genes in the isolates sequenced. A recent study found the size of the G genes from domestic turkeys varied depending upon the year of isolation and cell culture passage level. Early isolates had a large G gene with 1798nt bases, in agreement with those found in isolates from wild Canada geese (19), whereas later isolates possessed a smaller gene, consisting of just 783nt bases. After experimental passage in cell culture, larger genes became truncated, thereby resulting in a smaller gene. These findings suggest the contradicting reports on the length of subtype C were correct in their own right, however all recent isolates possess the larger G gene giving a total length of 14,150nt (228). Subtype C isolates have been reported to share 89-94% nt identity, compared with 60-65% with subtypes A and B (199).

Subtype D, which has very recently been fully sequenced in a complete MPV comparison study, was shown to have a genome length of 13,415bp and to be more closely related to subtypes A and B than to subtype C and hMPV throughout the majority of the genome (28).

2.2.5 Proteins

The AMPV genome encodes for eight structural proteins flanked either side by the 3' leader and 5' trailer ends, and follows the order 3'-N-P-M-F-M2-SH-G-L-5'. Members of the Pneumovirus genus express the same proteins however they also possess two non-structural proteins downstream of the 3' leader and follow a slightly different gene order of 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'.

The nucleocapsid (N), phosphoprotein (P) and large polymerase (L) are nucleocapsid proteins and together form the ribonucleoprotein complex, which is

surrounded by the matrix (M) protein layer. In association with viral RNA, this complex is responsible for RNA replication and transcription.

The attachment (G), fusion (F) and small hydrophobic (SH), proteins are transmembrane glycoproteins. For Pneumoviruses, it has been suggested that the G protein initiates attachment to the surface of host cells (139), whilst the F protein mediates fusion between the viral and host cell membranes. However recent studies, involving recombinant viruses with deleted genes, have led to the suggestion that the attachment and fusion processes may be more complex (79). The F protein of AMPV subtype A has been shown to be highly fusogenic compared with that of subtypes B and C (231). It is expressed as an inactive precursor F_0 which is cleaved by proteases, within the host cell, into two functional subunits F_1 and F_2. The precise function of the SH gene is not yet clear, however in AMPV subtypes A and B it is suggested to play a regulatory role in cell to cell fusion and to be important for replication *in vivo* and *in vitro* (146). A recombinant AMPV virus with a deleted SH gene resulted in reduced replication in cell culture and *in vivo,* and virus could not be not detected in the trachea of infected birds (146). In another recombinant virus study, AMPV lacking both the SH and G genes was shown to replicate in Vero cells, however it was slower than wild type virus and produced atypical CPE with unusually large syncytia (164).

The M2 gene consists of two overlapping open reading frames which express the M2:1 and M2:2 proteins. The M2:1 protein is important in transcription elongation and is thought to be associated with the nucleocapsid. It has been shown to enhance expression of FL mRNA in AMPV RG systems (55, 79, 164). The M2:2 protein, co-expressed in a HRSV minireplicon system, inhibited RNA synthesis and is thought to be involved in the switch between RNA transcription and replication (1, 55, 79, 111, 116).

2.2.6 Amino acid (aa) sequence identities

Proteins of subtypes A, B and D share a close nt and predicted aa sequence identity whereas subtype C is more closely related to hMPV. The N, P, M and F proteins of subtypes A and B have aa identities ranging from 72-91% (115, 140, 163, 187, 199) compared to 52-78% aa identity between subgroups A and C and subgroups B and C (196, 197, 199). Partial sequencing (323nt) of the F protein of subtype D revealed aa identities of 89.7%, 97.2% and 70.0% with subtypes A, B and C, respectively. The highly variable SH and G proteins share an aa identity of 47% and 38%, respectively, between subtypes A and B (115, 129) and 17.7-19.5% and 14-15.9% aa identity, respectively, for subtype C with subtypes A and B (101, 150). Subtype D shares 29.5%, 31.2% and 16% aa identity respectively with subtypes A, B and C within the highly variable G protein (16, 99). In the M2:1 and M2:2 proteins, aMPV A and B subtypes share aa identitys of 89% and 64% respectively, and together they share 70-71% and 20-21% aa identity respectively, with subtype C (73, 115). The L proteins of subtypes A and B share 85.5% aa sequence identity, and together share 62.5-64% aa identity with subtype C (150, 206). Subtype D shares 75.8% - 76.1% aa identity with subtype A in the initially determined L protein sequence (16).

2.2.7 Antigenic strain classification

AMPVs have been classified into subtypes A, B, C and D based on binding patterns of monoclonal antibodies (MAbs) in virus neutralization (VN) tests, subtype specific enzyme-linked immunosorbent assay (ELISA), and nucleotide sequence analysis heavily based in the G gene. Initial studies on the first AMPV isolates indicated a close antigenic relationship between those isolated from Britain and France (13, 93). Further studies, using MAbs and polyclonal antisera, found that British and French isolates formed a group (classified as subtype A) distinguishable from Hungarian, Italian and Spanish isolates (classified as subtype B) (53). Similar findings of distinct groups were seen in range of samples isolated in different years and originating

from different countries (62). These distinct groups, which belong to a single serotype, were named subtypes A and B after sequencing of the G protein revealed extensive variations (129).

Antigenic characterization tests on the first US isolate of AMPV subtype C revealed major differences compared with subtypes A and B. In cross neutralization tests, MAbs which neutralized subtypes A or B did not neutralize subtype C. Similarly, monospecific antisera to A or B didn't neutralize subtype C, and *vice versa* (60). Sequence analysis of the M protein also revealed significant differences between subtype C, and subtype A and B isolates (196). Subtype D isolates are antigenically distinct from subtypes A and B and the Colorado strain of subtype C, as shown by ELISA and VN tests (214). Sequence and phylogenetic analysis of the F, G and L genes of two French isolates led to the initial recognition and naming of subtype D (16).

2.3 Disease

2.3.1 Hosts

AMPV infects a range of avian hosts but is most notable in turkeys and chickens of all ages. Its role in chickens is more complicated then turkeys and it is often linked with SHS, however this condition can also be associated with other agents (89, 159). Both species are susceptible to subtypes A and B. Subtype C affects turkeys in the USA (198) and has recently been reported in chickens in China (196, 230). Subtype D has only been reported in turkeys (16).

Virus has been successfully isolated from naturally infected chickens (11, 34, 127, 136, 156, 182, 209) and turkeys (32, 33, 91, 125, 157, 174, 233, 238). Chickens can be infected naturally, and experimentally, with no sign of disease (33, 60, 65, 124, 180, 237). This can make isolation from chickens more difficult.

In other avian species, serological evidence of AMPV infection as shown by ELISA or VN has been detected in sea gulls (113), ostriches (35), guinea fowl (182) and pheasants (38, 96, 232). In the latter species mild respiratory disease was reported after experimental infections with a subtype A AMPV (94), and subtype C AMPV was successfully isolated in Vero cells from swabs taken in a live bird market in Korea (138).

In the USA, infectious AMPV has been isolated from Canada geese, and RNA, detected by RT-PCR found in wild birds including blue winged teals (20, 21), swallows, starlings, sparrows and geese (203). Antibodies to AMPV were identified by ELISA in American coots, American crows, Canada geese, cattle egrets and rock pigeons (220). In Brazil RNA was detected in pigeons, parrots and ducks such as the white cheeked pintail and the white faced whistling duck (84).

Infectious virus has been successfully isolated from commercial Muscovy ducks exhibiting respiratory problems and drops in egg production (208, 213) and from a sentinel mallard duck placed close to an AMPV infected turkey farm (203). In contrast, under experimental conditions Pekin ducks failed to develop clinical signs although the virus did provoke an immune response (202).

2.3.2 Experimental infections of chickens and turkeys

Under experimental conditions clinical signs have been reproduced in both turkeys (33, 34, 63, 68, 119, 125, 128, 136, 174) and chickens (8, 34, 37, 63, 64, 90, 124, 136, 153, 189, 201) though they are generally milder than those seen in the field (205).

Early experimental studies led to the suggestion that AMPV from chickens and turkeys possessed different biological behaviour *in vivo*. Disease in turkeys was seen following exposure to AMPV isolated from turkeys, whilst clinical signs in

chickens could not be reproduced with the same isolate (33, 94). An AMPV isolated from chickens was shown to induce disease in chickens and turkeys, though in the latter species disease was only mild (34). In a later study, chickens exhibited mild clinical disease following infection with the same turkey isolate used in the study by Buys et al (1989a) (33), but these signs were not reproducible in a second experiment (63). The author found clinical signs were more marked when each species was inoculated with a homologous AMPV isolate. A common finding in early experimental studies was that TRT strains isolated from turkeys could infect chickens but they didn't appear to cause disease in them (63). Whilst Jones et al (1987) were able to successfully infect chickens with a turkey-derived subtype A AMPV they only observed very mild clinical signs (124). However, in later studies clinical disease was successfully reproduced in chickens after inoculation with turkey-derived AMPV subtypes A, B and C (8, 189, 201).

Swollen sinuses, often observed in chickens during outbreaks of AMPV in the field, have been induced in some studies (8, 34, 182) whilst in others the condition was not seen (34, 37, 94, 124, 182).

It has been suggested differences such as the dose and virulence of virus strains, and the age, gender and immune status of birds may be a reason for varying outcomes between studies in chickens (8). Varying routes of virus inoculation may also be an important factor which could affect experimental results (64). Different local and systemic immune responses may be responsible for the differences in AMPV pathogenesis in chickens and turkeys (189).

2.3.3 Relationship between AMPV and hMPV

hMPV is associated with acute respiratory tract infections and causes clinical symptoms similar to HRSV, ranging from mild respiratory problems to severe bronchiolitis and pneumonia. It was initially believed to be a novel virus until serological studies of archived samples revealed it had been circulating in humans

for at least 50 years (224). It is distributed worldwide and affects all age groups, although it occurs more often and with higher severity in young children, elderly adults and the immunocompromised (26).

Phylogenetic and sequence analysis has revealed two major genotypes, A and B, (12, 181, 224) which have been further divided into four sub-lineages A1, A2, B1 and B2, based upon sequence analysis of the F and G genes (225). hMPV is more closely related to AMPV subtype C than to any of the other AMPV subtypes. In the N, P, M, F, M2-1 and M2:2 genes they share an overall aa identity of 80%, a percentage range seen between other closely related subtypes AMPV A and B, and RSV A and B (223). Within the SH and G genes however, the similarity is low, with a predicted aa identity of < 30% (150). It has been suggested that this low identity may explain the different host range (223). However a recent study suggests that the F protein may be the main determinant for the host tropism of metapneumoviruses (75).

It has been speculated that hMPV originated in birds and jumped species into humans around 200 years ago (74). Experimental infections of turkeys with hMPV have produced contradicting outcomes. Velayudhan et al (2006) induced clinical signs in two-week-old turkeys similar to those seen in birds experimentally infected with AMPV subtype C (227) whereas in a similar study clinical signs were not seen in either turkeys or chickens (224, 227).

2.3.4 Transmission

Although AMPV has been detected in the reproductive tract of laying birds (128, 135) and infection in young turkey poults has been reported (95, 200), there is no firm evidence that infectious virus can be transmitted vertically from adult birds to offspring.

Transmission is most likely horizontal from aerosols or contaminated equipment and organic materials. To date there is no clear information on whether horizontal transmission occurs specifically via the respiratory route, the oral route, or both. Evidence of transmission between infected and uninfected birds placed in direct contact has been reported (4, 68, 91, 125, 174). An attenuated candidate vaccine virus was shown to spread from inoculated to susceptible contact birds and also to induce protection (234). Transmission by indirect contact has been reported. Virus was found to have been transmitted between turkeys housed in neighbouring cages (92) whilst RNA and AMPV antibodies were detected in caged sentinel mallard ducks which had been placed in close proximity to AMPV infected turkey farms (203). In contrast, Cook et al (1991) found AMPV did not transmit from infected to uninfected birds housed in separate cages but within the same room (68), as did Alkahalaf et al (2002) who performed a similar study with subtype C (4).

Wild birds have been implicated as a possible transmitter, since virus has been detected in many species. Virus has been detected in wild birds in states lying south of Minnesota (Georgia, South Carolina, Arkansas, Ohio) and it has been suggested they potentially provide a mechanism to spread virus to poultry in other regions of the USA, and possibly to countries within Central and South America (220). However, there is no evidence of subtypes A and B spreading north to the USA from Central and South America, or of AMPV in Canada, in spite of the migratory wildfowl flyways which span the continent.

2.3.5 Geographical Distribution

The first reported case of AMPV was detected in 1978, from turkey flocks in South Africa (32) followed by reports from Britain (2, 6) and France (92). The disease now affects gallinaceous birds on most continents (25), amongst which countries include Spain, Germany, Hungary, Netherlands, Israel (62, 166), Morocco (80), the USA,

Mexico, Chile, Brazil (7, 76, 121, 191, 198, 218), Japan, Korea and China (136, 208, 209, 230). Canada and Australia are presently unaffected (18, 112).

Initial studies suggested that the first AMPV isolates from South Africa, the UK and continental Europe were antigenically related, although they formed two distinct types. South African, UK and French isolates were placed within subtype A, and continental European isolates from countries such as Hungary, Spain and Italy within subtype B (53, 62, 129). Between 1985-1990 all UK AMPV isolates were identified as subtype A, however a survey of turkey flocks in North, West and Eastern Britain between 1994 and 1995 revealed subtype B was largely present (161) . Conversely, in continental Europe all isolates were classified as subtype B, until 1998 when subtype A was identified in Belgian turkey farms (221). However more recently Hafez et al (2000) determined AMPV isolates from early clinical outbreaks in Germany were subtype A (109), and hence both subtypes had been present in continental Europe during the late 1980s.

The USA has only been affected by AMPV subtype C. Infection was first detected in Colorado in 1996 and was successfully eradicated after 10 months due to strict biosecurity measures (198). Shortly afterwards the disease appeared in Minnesota, in counties highly populated with commercial turkeys (151). However, here too the disease appears to have been eradicated (123). ELISA and/or RT-PCR analysis showed presence of the disease in turkeys, in the neighbouring states of North Dakota, South Dakota, Iowa and Wisconsin (21).

Reports of subtype C detected outside the USA come from Muscovy ducks in France (213) which belong to a different genetic lineage, (214, 217), from Muscovy ducks and chickens in China (208, 230) and from pheasants in Korea (138).

2.3.6 Clinical disease

Clinical disease associated with AMPV infection can vary in severity in turkeys. Younger birds are more susceptible and the virus has been reported in poults as young as 5 days old (95). Environmental factors such as poor hygiene and overstocking can exacerbate the disease (205) as can secondary bacterial infections (68). Disease can spread through a flock within 24 hours (205). Typical clinical signs include sneezing, tracheal râles, nasal discharge, foamy conjunctivitis, swollen infraorbital sinuses, head shaking and coughing (57, 166). Birds can recover within 10-12 days, under good management conditions and in the absence of secondary infections (33, 125, 157, 238). In laying birds a drop in egg production and egg shell quality is common (70, 205) and recovery can take up to 3 weeks (57). Morbidity can reach up to 100% in birds of all ages, but mortality rates are variable, and in complicated cases have been reported to exceed 50% (6, 205).

In chickens, the virus affects both broilers and broiler breeders and is often associated with SHS (237). However AMPV infections have been detected in birds without SHS (65), and the condition has been linked to other respiratory pathogens such as infectious bronchitis virus (IBV) (78, 159). Typical symptoms of SHS include swelling of the periorbital and infraorbital sinuses, torticollis, cerebral disorientation and opisthotonous. Other signs include coughing, sneezing, eye and ear discharge, red conjunctivitis, severe tracheitis and foul-smelling green diarrhoea, and birds can die as a result of secondary infection with *E.coli*. A decrease in egg production is seen in breeders. Mortality and morbidity rates are generally low and disease generally lasts for 2-3 weeks (159, 170, 180). Co-infection with other agents and poor environmental conditions appear to be important factors in the development of typical SHS symptoms.

2.3.7 Pathogenesis

(i) Replication and persistence

AMPV virus replication and distribution in the tissues is similar for chickens and turkeys (57). Replication takes place mostly in the upper respiratory tract, in the nasal turbinates, trachea and lungs. Following experimental infections, viral antigen has been detected by immunoperoxidase (IP) and immunoflourescence (IF) in the ciliated epithelium of the nasal turbinates between days 2 and 6 post infection (pi), and of the trachea between 1 and 7 days pi (37, 90, 125, 128, 153). Following intranasal and eye-drop inoculation of turkeys, antigen was detected in the ciliated surface of bronchial epithelial cells 6 days pi and in chickens between days 3 and 4, but only after intratracheal, intranasal and eye drop inoculation (153). Intense staining was observed at 3 and 5 days pi in the conjunctival epithelium of chickens inoculated with a chicken derived subtype B isolate (90), whereas this was not reported in a similar study (37).

From 2 to 5 days pi large amounts of virus can be recovered from the nasal turbinates and tracheas, and afterwards in much smaller quantities up to 14 days after inoculation (37, 63, 68, 128). Virus has been recovered from the lungs of infected turkeys and chickens from 5 to 7 days and 1 to 6 days pi respectively (37, 68) and from the air-sacs of turkeys between 3 and 11 days pi (68). Inoculation of virulent AMPV together with *Bordetella avium* or *Pastuerella*-like microorganisms increased the invasiveness (4, 68) and virus was recovered in small amounts from internal organs (68). In contrast, during dual infection of turkeys with AMPV and *Mycoplasma gallisepticum* (Mg) there was no apparent increase in virus persistence or invasiveness (162).

The virus has been shown to replicate in the reproductive tract of laying turkey hens (128, 172). Jones et al (1988) detected viral antigens in the uterus epithelium on day

7 pi, and the uterus and all other regions of the oviduct on day 9 pi (129). In addition it was isolated, following 3 passages in TOC, from the middle magnum and the vagina 9 days pi. At present there are no reports of AMPV virus replication in the reproductive tract of chickens.

With the use of PCR, viral RNA has been detected in tracheal swabs from turkeys infected with subtype A for up to17 days pi (118) and was present in respiratory tissues for 9 days, and the blood for 15 days, after inoculation of turkeys with a subtype C isolate (201). In chickens, viral RNA was detected up to 11 days after exposure to subtypes A and B (8). Subtype B was reported to have a wider tissue distribution, and persist for longer in chickens than subtype A (8, 189).

(ii) Macroscopic lesions

Following field outbreaks, where complicating secondary pathogens are often involved, *post mortem* lesions including pneumonia, pericarditis, airsacculitis and perihepatits have been described in turkeys and chickens (89, 119, 148, 205, 221). Opaque air sacs and calluses in the lungs were found after an outbreak on a Belgian turkey farm (221). Major gross lesions in chickens include extensive yellowish gelatinous to purulent odema in the subcutaneous tissues of the head neck and wattles (89, 148).

AMPV infection of 5 week old turkeys, under experimental conditions caused complete deciliation of the trachea 96 hours pi (125). In laying turkeys, a watery to mucoid exudate was detected in the turbinates, and excess mucus in the trachea (128). Severe peritonitis was reported by Cook et al (2000) (64), and by Jones et al (1988) who described various other lesions of the reproductive tract (128).

(iii) Histopathology

Histopathological lesions of the nasal turbinates, trachea, and less often, the lungs have been described following experimental infections of chickens (8, 37, 124, 153, 189) and turkeys (119, 124, 125, 153, 154, 174, 226) and are similar in the two species.

Initial changes seen at 1 or 2 dpi include an increase in glandular activity, focal loss of cilia, and mild mononuclear infiltration of the submucosa. By day 4 to 5 copious mononuclear inflammatory infiltration can be seen in the submucosa together with focal discontinuity of the epithelial layer. By days 6 to 10 pi lesions are more advanced and mucus and epithelial cells can be seen in the lumen. Mild lesions have been detected in the submucosa of secondary bronchi (8, 153). Infiltration of lymphocytes and development of lymphoid follicles were observed in the Harderian glands of broilers, infected with subtypes A and B, at 6 and 11 days pi (8, 189) and in turkeys infected with subtype C (49). Active regeneration can be seen at 10 days pi and tissues have recovered between 14 and 21 days pi.

2.4 Immunity

(i) Humoral immunity

Humoral antibody responses have been detected with the ELISA, VN and IF tests (14, 15, 50, 65, 103, 171). Following natural infection of turkeys Baxter-Jones et al (1989) detected antibodies by all three serological methods, 5 days after the appearance of clinical signs (14). VN antibodies quickly reached peak titres and were in decline by day 13, as were IF titres, whilst ELISA antibodies peak titres were achieved at day 13 and persisted for at least 34 days. The appearance of VN antibodies before ELISA antibodies agrees with the findings of others following experimental infections of chickens and turkeys (128, 142, 189, 193) and despite the

delayed reaction good correlation has been reported between the two serological methods (14, 65, 128, 189).

During an experimental infection of mature laying turkeys, VN antibodies titres reached $log_2 10$ by day 7 pi, 2 days before the same level was reached by ELISA antibodies. By 12 days pi high titres were reported for both and remained high throughout the 89 day study. The high antibody titres and their prolonged persistence agreed with field observations that higher antibody levels are seen in sexually mature turkeys than in younger birds (128).

(ii) Cell mediated immunity

Despite the clear humoral antibody responses observed in chickens and turkeys, evidence suggests they don't play a major role in the protection against AMPV infections and that cell mediated immunity may be more important. Vaccinated turkey poults which were unable to produce humoral antibodies, due to chemical bursectomisation, were still resistant to challenge with virulent virus (126) and in a study by Williams et al (1991b) inoculation with a Vero cell attenuated virus prompted very little antibody response in young poults, yet they were protected against challenge (235). In addition, slightly older birds that had no circulating antibodies at the time of challenge were protected for up to 22 weeks. Conversely, high levels of passively transfered antibodies, detected by ELISA, did not offer protection to turkeys challenged with a virulent AMPV (193) nor did maternally derived antibodies (169).

The importance of T lymphocytes in the control and clearance of AMPV has been highlighted (192). T- cell suppression in turkeys following treatment with Cyclosporin A resulted in a slower recovery from clinical signs and histopathological lesions, and prolonged presence of viral RNA following inoculation with virulent virus. The humoral antibody response was not compromised.

(iii) Local immune response

The local immune response is stimulated during infection with AMPV, when viral antigens come into contact with target mucosal cells. The secretory immunoglobulin IgA is a major local antibody and, with the use of the ELISA, has been detected in the lachrymal fluid of experimentally vaccinated chickens (88), and in tracheal washings of chickens infected with virulent subtypes A and B (189). Khehra, R. S (134) detected IgA in lachrymal fluid and tracheal washings from both chickens and turkeys. It has also been demonstrated in nasal washes and bile of turkeys infected with subtype C (46).

Evidence suggests that local humoral immunity, which is likely to be important in the defence against AMPV, is short lived. Local IgA antibodies and VN antibodies, detected in tracheal washes of chickens, declined after 10-11 days pi and 14 days pi respectively (189). In turkeys infected with subtypes A and B, VN antibodies detected in tracheal washes peaked between 7-14 days pi followed by a gradual decline (142).

2.5 Diagnosis

2.5.1 Clinical signs

Diagnosis of AMPV infection cannot be made from clinical signs alone. Other infections such as Mycoplasma, influenza and Newcastle disease can cause disease similar to AMPV (166). In addition chickens can be infected without showing signs of disease (65). Antibody or virus detection is required for confirmation.

2.5.2 Virus isolation and demonstration

The isolation of virus from turkeys and in particular chickens is made difficult due to the short presence of the virus following infection. Samples from the nasal tissue or

trachea should be taken at the very first sign of clinical disease, however once clinical signs are obvious there will be very little virus remaining in the bird (58).

For the primary isolation of AMPV, chicken or turkey TOC, as described by Cook et al (1976) (59), or embryonated eggs are commonly used. Many early subtype A and B viruses were isolated using TOC (33, 92, 95, 125, 157, 233, 238), and cilostasis has been observed as early as 4 to5 days pi. However further passages may be needed if viral titres are low (58). Williams et al (1991b) suggested TOC passage may simulate bird-to-bird passage after a subtype A isolate was shown to retain its virulence following 98 passages (234) Ciliostasis is not seen in TOC inoculated with subtype C AMPV therefore this method is not suitable for the primary isolation of US isolates (60). Embryonated eggs inoculated via the yolk sac were used to isolate the first AMPV from South Africa (33) and one of the first subtype C isolates from the US (174).

Cell culture systems including chicken embryo related (CER) cell lines, continuous quail tumour cell lines (QT-35), chick embryo fibroblasts (CEF), and CEL have also been used for the primary isolation of AMPV (20, 71, 91, 102, 110, 119). The African green monkey kidney cell line (Vero) has been used successfully for subtype A and B following a process involving multiple serial trypsinisation and cell splits (9, 136).

Once isolated, virus has been adapted to grow in CEF, CEL and more commonly in Vero cells (33, 103, 174, 234). Cell culture systems can quickly lead to the attenuation of the virus, although in one study multiple passage in TOC did not lead to the attenuation of a subtype A field strain. (234). Vero cells have been used to attenuate AMPV during the development of commercial vaccines (177, 234, 235).

Immunoflourescence (IF) (15, 125, 153), immune-peroxidase (IP) staining (153, 172), immunogold staining (173) and electron microscopy (33, 92, 157, 238) have been used to demonstrate the presence of virus in fixed and unfixed tissue samples and smears. RT-PCRs are widely used, and are less time consuming than other techniques making it relatively easy to screen large numbers of samples (41). They are highly sensitive and very small quantities of viral RNA can be detected, and for longer periods, than infectious virus or antigens. A range of RT-PCR assays have been developed using primers specific to the N, M, F and G genes, however only amplification of the N gene enables the detection of all four known AMPV subtypes (17, 213). Subtypes A and B can be detected and differentiated using G gene specific primers (41), while amplification of the F or M genes allow specific detection of subtype C (195, 204). Real time RT-PCR assays have been developed which can detect subtypes A and B (45, 86, 136) and subtype C (226). A protocol also exists which can detect all four subtypes (104).

2.5.3 Serology

Serological methods can be used to detect antibodies circulating in sera and respiratory secretions following exposure to AMPV. The IF test performed in TOC was used to detect AMPV antibodies, but the method was found to be labour intensive and impractical for large numbers of samples (14). However, an IF test to detect subtype C AMPV antigen in Vero cell cultures proved to be sensitive and specific, and enabled the detection of viable virus in 24 hours pi (120).The virus neutralisation test can be performed in TOC, CEF, CEL, MA104 and Vero cells (3, 14, 171, 214) It is a sensitive and simple method, and has good correlation with ELISA and IF (3, 14), although it is a time consuming and laborious test for routine lab diagnosis.

The ELISA is the most commonly chosen method and has been developed for in-house and commercial use (14, 50, 51, 81, 103, 171). The subtype of the ELISA coating antigen has been shown to be an important factor in the detection of AMPV antibodies. It has been reported that vaccinal antibodies may not be detected if the ELISA coating antigen is a heterologous strain and suggested that homologous antigens should be used (81). In agreement subtype A and B antigens were shown to be very poor for the detection of subtype C antibodies (60). ELISAs for the detection of subtype C have since been developed using homologous strains (3, 51, 152). Furthermore, ELISAs for the detection of AMPV C have been developed in which recombinant M or N proteins are used. Whilst both were reported to be highly sensitive and specific, the N protein was reported to be more so than the routine indirect ELISA (105, 106).

2.6 Control

2.6.1 Management and treatment

The risk of AMPV infection may be reduced and effects minimised if good farm management and bio-security practices are employed. Disease can be exacerbated by factors such as inadequate ventilation and temperature control, poor litter quality and general hygiene, high stocking densities, multi-age stock and secondary pathogens (122, 135, 147, 205). It has been recommended that all delivery and catching crews, equipment, and feed trucks should be routinely disinfected (97).

Whilst there is no effective treatment for AMPV infections, secondary bacterial infections may be controlled with the use of antibiotics (108).

Recent studies using RNA interference (RNAi) have described the inhibitive effect on AMPV replication, *in-vitro*, of double stranded short interfering RNA molecules (siRNA) for the N gene of subtype A and the P gene of subtype C (85, 160). These,

and future findings may lead towards the development of novel antiviral therapies for AMPV.

2.6.2 Vaccines

Commercially available attenuated live and inactivated vaccines are currently the most effective tool used in the control of AMPV infections in turkeys and chickens. Live vaccines have been developed by the adaption and attenuation of virulent AMPV in embyronated eggs (33), in Vero cells or CEF (33, 107, 178, 179, 234, 235), following alternate passages in TOC and chick embryos (66, 67, 69) and in Vero cells adapted to grow in sub-optimal temperatures (175, 177). Experimentally, live attenuated vaccines have been shown to offer full protection against homologous challenge (61, 67, 81, 176, 234, 235) and subtype A and B vaccines can offer good cross protection against heterologous challenge (61, 81, 161, 216, 222). Both subtype A and B vaccines can also protect against AMPV subtypes C (60) and D (57). Following vaccination with a subtype C Colorado isolate some protection was seen in turkeys challenged with subtype A but not following challenge with subtype B (60).

The humoral antibody response induced by live vaccination both experimentally and in the field can be poor, yet birds can still be protected (70, 97, 176, 235). The measurement of AMPV antibodies following vaccination cannot be an indicator of the efficacy of a vaccine (193, 235).

Inactivated vaccines induce a good antibody response (70, 158) however their ability alone to protect birds is uncertain. Two recent experimental studies using subtype C inactivated vaccines gave conflicting results. Kapczynski et al (2008) found an inactivated AMPV-C vaccine failed to protect turkeys against experimental challenge, reduce virus shedding or decrease histopathological lesions (132), whilst

in another study inactivated AMPV-C vaccine, administered via the same route was shown to induce protective immunity (47). Some protection against the effect of AMPV on egg production has been reported but a programme of vaccination with both live and inactivated vaccine is recommended to give complete protection to adult birds (70, 97).

Experimental vaccinations using live attenuated strains administered *in ovo* have been reported in turkeys and chickens (210, 236). In the field live vaccines are routinely applied via coarse spray but can be effective when administered via the drinking water (87, 97). Stringent vaccination is required for large flocks in a bid to ensure every bird is exposed and receives an efficacious dose. A major downside to live attenuated vaccines is their genetic instability. A commercially available subtype A vaccine has been shown to revert to virulence in the field and the authors suggest unvaccinated birds within a flock may passage virus shed from vaccinates until the virus mutates (36). With the use of a RG system, studies on this revertant strain revealed that one base mutation out of five identified was found to be responsible for the reversion to virulence (29). In a recent study, virulent virus causing disease in a poultry flock in Italy was found to have originated from the same subtype A vaccine. However this had not been applied to birds on the site for at least 6 months suggesting that the revertant had either been persisting in the environment or had been brought in via other sources (149). More recently the reversion to virulence of a subtype B vaccine has been reported (44).

Current live vaccines may fail to offer continued protection against new and evolving strains of AMPV (10, 39) and vaccine breaks are often observed in the field (11, 221). Mass live vaccinations may be a factor driving the evolution of the virus (43). Recent research has focused on the development of improved stable vaccines for better control or eradication of AMPV. Recombinant subunit and DNA vaccines have

been developed, but have only been shown to offer partial protection against challenge. These include a fowl pox virus vector containing the AMPV F protein (185), virosome vaccines containing the F or G proteins (131) and DNA vaccines containing the F or N proteins (133, 211). In addition Liman et al (2007) developed a prime-boost method in which turkeys were primed with microparticles carrying plasmid DNA encoding for the F protein, then boosted with a micro-encapsulated, homologous recombinant F protein (141). In recent findings recombinant methyltransferase-defective AMPV subtype C Colorado strains were found to be highly attenuated in turkeys and to provide complete protection against challenge with homologous and heterologous AMPV subtype C strains (207).

2.7 Reverse genetics studies

RG systems have been developed to allow the manipulation of many Paramyxoviruses, including HRSV, BRSV, Human parainfluenza virus 3, Newcastle disease virus (NDV), Rinderpest virus, Sendai virus, Simian virus 5 (155), hMPV (24) and AMPV (98, 164). These systems are an important tool for future live vaccine development.

In AMPV RG studies, specific base mutations, or the modification or deletion of entire genes has been enabling studies into factors such as virulence , protection and virus growth properties, both *in vitro* and *in vivo* (29, 146, 164, 168, 242). In addition, an AMPV subtype A and B positive control system, for use with diagnostic RT-PCRs, has been developed using a RG approach (83).

(i) Deleted M2:2 protein recombinant viruses

The generation of recombinant viruses with deleted M2:2 proteins, and the effect on replication *in vivo* and *in vitro* have been described for AMPV (146, 242), hMPV (23, 31, 194) and RSV (22, 56, 116, 117, 212).

The M2:2 protein appears to play an important role during replication *in vivo* but may not be essential for growth *in vitro*. A recombinant subtype A AMPV which lacked the M2:2 protein replicated slightly less efficiently in cell culture than wild type virus, but was more severely impaired in turkeys (146). Similarly, an M2:2 deleted subtype C AMPV led to inhibited replication in turkeys, while growth in Vero cell culture was not significantly affected. In this study less than half of the turkeys developed a positive antibody response following inoculation with the recombinant virus, and it failed to confer protection against challenge to the majority of the birds. The authors suggested the virus may have been over attenuated (242). In contrast, a recombinant RSV with a deleted M2:2 protein resulted in highly inhibited replication in the respiratory tracts of cotton rats and mice yet conferred protection against challenge with wild type virus. In vitro, growth was impaired in HEp-2, HeLa, and MRC5 cells but was efficient in Vero and LLC-MK2 cells (116). Similarly, a hMPV recombinant was shown to provide protection against challenge despite reduced replication in the lungs of hamsters. Plaque size was markedly smaller in Vero cells but viral titre was relatively unaffected (194).

Chapter 3

General materials and methods

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Chapter 3

General materials and methods

This chapter describes general materials and methods used throughout the study.

3.1 Glassware and instruments

Glassware was machine washed, rinsed in ultra-pure water and autoclaved for 30 minutes at 121^{o}C. Measuring cylinders were sealed with aluminium foil caps and sterilised at 160^{o}C for two hours in a hot air oven. Instruments for TOC were sealed in pouches, autoclaved with glassware and dried in a hot air oven.

3.2 RNA extraction and Reverse Transcription

3.2.1 Viral RNA extraction

Viral RNA was extracted and purified using a QIAamp Viral RNA spin kit [1], according to the manufacturer's protocol.

3.2.2 Reverse Transcription (RT)

Extracted RNA was reverse-transcribed using Super Script™ II Reverse Transcriptase [2]. This was added to the reaction at 42^{o}C to avoid oligo mispriming.

(i) RT reaction mix

5 x First-Strand Buffer	4µl
DTT (0.1M)	2µl
dNTP solution (40mM)	1µl
Forward primer (10µM)	1µl
Extracted RNA	2µl
Rnasin [3] (40U/µl)	0.5µl
Water [4]	to 20µl

[1] Qiagen Cat. no. 52904 QIAamp Viral RNA kit
[2] Invitrogen Cat. no. 18064-014 SuperScript™ II ReverseTranscriptase
[3] Promega Cat. no. N2111 Rnasin® Ribonuclease Inhibitor
[4] Sigma Cat. no. W3500 Double processed tissue culture water

Mineral Oil [5]	50µl
SuperScript II RT (200U/µl)	1µl

(ii) RT cycle

(1)	70°C	1 minute
(2)	50°C	2 minutes
(3)	42°C	10 seconds
(4)	42°C	Hot start
(5)	42°C	1 hour 30 minutes
(6)	94°C	1 minute
(7)	12°C	Hold

3.3 Polymerase Chain Reaction (PCR)

Different polymerases were used in PCRs depending upon the required amplicon size or subsequent downstream applications. BIO-X-ACT Long DNA polymerase [6] was used to generate PCR products from cDNA. Blunt ended products were generated by PfuTurbo DNA polymerase [7] for use in site-directed mutagenesis (SDM), ligations and overlap PCR's while general short screening PCRs were performed using GoTaq Flexi DNA polymerase[8]. To avoid oligo mispriming BIO-X-ACT and Pfu Turbo were added to the reactions at 80°C.

3.3.1 BIO-X-ACT (a) and Pfu Turbo (b) reaction mix

	(a)	**(b)**
10x Buffer	5µl	5µl
$MgCl_2$ solution (50mM)	2µl	-
dNTP solution (40mM)	1µ	1µl
Forward primer (10uM)	1µl	1µl
Reverse primer (10uM)	1µl	1µl
Template (plasmid DNA 120ng/µl)	1µl	1µl
BIO-X-ACT (4U/µl)	0.8µl (add at 80°C)[6]	-

[5] Sigma Cat. no. M8410 Mineral oil
[6] Bioline Cat. no. BIO-21049 BIO-X-ACT™ Long DNA Polymerase
[7] Stratagene Cat. no. 600252 PfuTurbo® DNA Polymerase
[8] Promega Cat. no. M8305 GoTaq® Flexi DNA Polymerase

Pfu Turbo DNA Polymerase (2.5U/µl)[7]	-	1µl (add at 80°C)
Tissue culture water [9]	38µl	40µl
Mineral oil [10]	50µl	50µl

(i) BIO-X-ACT and Pfu Turbo cycle

(1)	80°C	10 seconds
(2)	80°C	Hot start
(3)	94°C	5 seconds
(4)	50°C	20 seconds
(5)	68°C	1 minute / Kb
(6)	Repeat steps 2 to 4 for 5 cycles	
(7)	Repeat steps 2 to 4 for 25 cycles with a 10 second time increment	
(8)	12°C	Hold

3.3.2 GoTaq reaction mix

5x GoTaq Flexi Buffer	5µl
$MgCl_2$ solution (25mM)	1.75µl
dNTP solution (40mM)	0.5µl
Forward primer (10uM)	0.5µl
Reverse primer (10uM)	0.5µl
Template (cDNA)	2µl
Tissue culture water[11]	to 25µl
GoTaq DNA Polymerase (5U/µl)[12]	0.25µl
Mineral oil[13]	50µl

(i) GoTaq Cycle

(1)	94°C	15 seconds
(2)	94°C	10 seconds

[9] Sigma Cat. no. W3500 Double processed tissue culture water
[10] Sigma Cat. no. M8410 Mineral oil
[11] Sigma Cat. no. W3500 Double processed tissue culture water
[12] Promega Cat. no. M8305 GoTaq® Flexi DNA Polymerase
[13] Sigma Cat. no. M8410 Mineral oil

(3)	42^{o}C	20 seconds
(4)	72^{o}C	40 seconds
(5)	Repeat steps 2 to 4 for 35 cycles	
(6)	8^{o}C	Hold

3.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise and confirm the size of PCR and SDM products, and for the quantification of plasmid DNA. Concentrations ranging from 0.8% to 2% w/v were prepared in TBE buffer[14] which was diluted 10 fold before use (appendix). Red Safe Nucleic Acid staining solution[15] was added during preparation of the gel to allow product visualisation under U.V light. Sample (10 µl) was mixed with 4µl of load buffer (appendix) before loading onto the gel. Molecular weight markers (MW) λDNA/*Hin*d III[16] and ΦX174 RF DNA/*Hae* III[17] were used to determine product size (appendix).

3.5 Sequencing

PCR products were treated with Shrimp Alkaline Phosphatase[18] (SAP) and Exonuclease I[19] (EXO) to dephosphorylate and degrade residual dNTPs and primers (appendix), and submitted to Beckman Coulter Genomics. Data was aligned and analysed using Chromas (version 1.45), Generunner (version 3.05) and Bioedit Sequence Alignment Editor (version 7.0.9.0).

3.6 Ligations

Plasmid circularisation, and ligation of DNA sequence with plasmids, was performed

[14] Invitrogen	Cat no. 15581-044	Ultrapure TBE buffer, 10X
[15] Intron Biotechnology	Cat. no. 21141	Red Safe Nucleic Acid staining solution
[16] Invitrogen	Cat.no 15612-013	λDNA/*Hin*d III
[17] Invitrogen	Cat no. 15611-015	ΦX174 RF DNA/*Hae* III
[18] Usb	Cat. no. 70092Z	Shrimp Alkaline Phosphatase
[19] Usb	Cat. no. 70073Z	Exonuclease I

using T4 DNA Ligase[20]. Reactions followed the supplier's protocol. Xho I[21] and Sal I[22] RE were added to the mixture to prevent self ligation. Xho I and Sal I RE have different recognition sequences but have the same 5' TCGA overhang. After digestion the two different sites can be ligated. This leads to a sequence that does not contain the complete recognition sequences of either RE, Xho I or Sal I, and therefore cannot be digested by them.

3.7 Site-Directed Mutagenesis (SDM)

SDM was performed using primer pairs or blunt-ended PCR amplicons (mega primers) to introduce single or multiple nt substitutions to non-methylated cDNA. Original, methylated plasmid templates were then digested using *Dpn* I[23] to render them non-viable upon *E.coli* transformation (figure 1). For SDM employing PCR amplicons as mega primers, 5µl of amplicon (3-4ng/µl) was used and the volume of tissue culture water adjusted to give a final volume of 50µl.

(i) SDM reaction mix

10x PFU Buffer	5µl
dNTP solution (10mM)	1µl
Forward primer (10uM)	1µl
Reverse primer (10uM)	1µl
Plasmid DNA template (120ng/µl)	1µl
Tissue culture water [24]	40µl
Mineral oil [25]	50µl
Pfu Turbo DNA polymerase[26]	1µl (add at 80^{o}C)

[20] Fermentas	Cat.no. EL0013	T4 DNA Ligase
[21] Promega	Cat. no. 15217-011	Xho I
[22] Invitrogen	Cat. no. R6161	Sal I
[23] Invitrogen	Cat. no. 15242-019	*Dpn* I
[24] Sigma	Cat. no. W3500	Double processed tissue culture water
[25] Sigma	Cat. no. M8410	Mineral oil
[26] Stratagene	Cat. no. 600252	PfuTurbo® DNA Polymerase

(ii) SDM cycle

(1)	80^{o}C	10 seconds
(2)	80^{o}C	Hot start
(3)	94^{o}C	30 seconds
(4)	94^{o}C	30 seconds
(5)	55^{o}C	1 minute
(6)	68 ^{o}C	30 minutes
(7)	Repeat steps 3 to 5 for 18 cycles. Hold at 12^{o}C	

(iii) *Dpn* I digests

SDM product (10µl) and 0.5ul *Dpn* I[22] (10U/µl) were incubated for a minimum of two hours at 37^{o}C.

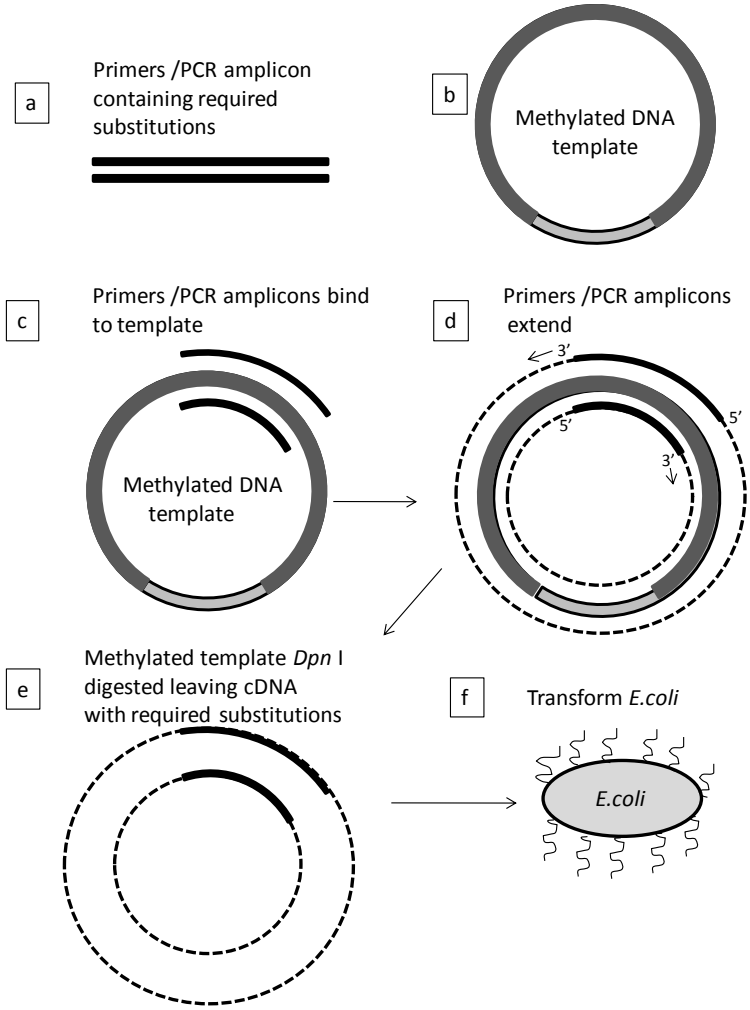

Figure1. SDM, using either primers or high fidelity blunt ended RT-PCR amplicons, introduces required substitutions to cDNA. PCR amplicons or primers containing substitutions (a), and methylated DNA template (b) are combined in an SDM reaction (c). The amplicons / primers extend, copying the template (d), which is later digested by *Dpn* I (e). The substituted cDNA is then used to transform *E.coli* (f).

3.8 *E.Coli* transformations

MAX Efficiency Stbl2 Competent Cells [27] were transformed with constructs from SDM and ligation reactions.

SDM or ligation product (1µl) was gently mixed with 70-100µl Stbl2 cells and incubated for 30 minutes on ice, heat shocked at 42^{o}C in a water bath for 25 seconds and returned to ice for a further two minutes. SOC medium (500µl) was added and samples agitated and incubated at 25^{o}C for 90 minutes. A volume of 100-200µl of transformed cells were inoculated onto LB agar plates, containing Kanamycin antibiotic at a concentration of 15 µg/ml, and incubated at 30^{o}C overnight.

(i) Liquid cultures

A single colony was added to 15 ml LB broth [28] containing Kanamycin at a concentration of 15µg/ml. Cultures were agitated at 25^{o}C for 24 to 48 hours.

3.9 Plasmid purification

Plasmid DNA was purified from 500µl of liquid culture using a QIAprep Spin Miniprep Kit[29], following the supplier's protocol. Plasmid DNA was eluted in water[30] and stored at -20^{o}C.

3.10 Restriction enzyme digests

Restriction enzymes (RE) *Eco* RI [31] and *Bam* HI [32] were used to digest purified plasmid DNA for restriction mapping and for plasmid quantification. Xho I[33] or

[27] Invitrogen	Cat. no. 10268-019	Stbl2 Competent Cells
[28] Gibco	Cat.no. 10855	LB Broth
[29] Qiagen	Cat. no. 27106	QIAprep Spin Miniprep Kit
[30] Sigma	Cat. no. W3500	Double processed tissue culture water
[31] Invitrogen	Cat. no. 15202-013	*Eco* RI
[32] Invitrogen	Cat. no. 15201-023	*Bam* HI
[33] Promega	Cat. no. 15217-011	*Xho* I

Sal I[34] were used to prepare PCR products and plasmid DNA for sticky end ligation. Digestions followed the supplier's protocol.

[34] Invitrogen Cat. no. R6161 *Sal* I

Chapter 4

Molecular comparison of full-length genome sequences of subtype A and B avian metapneumoviruses of chicken and turkey origin.

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Chapter 4

Molecular comparison of full-length genome sequences of subtype A and B avian metapneumoviruses of chicken and turkey origin.

4.1 Introduction

To undertake this comparison study a FL RT-PCR and sequencing system was first developed for the subtype B viruses. A large majority of subtype B sequence had been successfully obtained during a previous project. Existing subtype A and B primers that corresponded well with this sequence were used to obtain an unknown FL sequence of a commercial subtype B vaccine. New primers were then designed based on this sequence to create an efficient subtype B specific protocol. A system for subtype A AMPVs had previously been developed at the University of Liverpool.

The newly developed subtype B and the existing subtype A FL RT-PCR protocols were then used to obtain the complete genome sequences from chicken and turkey-derived AMPV vaccine and field strain viruses. Circularisation of RNA was employed to determine the precise sequence of the extremities. Nucleotide and predicted aa sequence alignments were then used to look for potential species specific motifs, which may be of importance for host range and ultimately may support the notion that homologous vaccines have greater efficacy.

Finally, a parallel study was undertaken where turkey-derived AMPVs of known sequence were passaged ten times in chicken TOC , then re-sequenced and compared to the original. This was performed in an attempt to generate potential species specific motifs.

4.2 Materials and methods

4.2.1 Determining the full length sequence of a commercially available subtype B vaccine.

Viral RNA was extracted (3.2.1) from a subtype B commercial vaccine and reverse transcribed (3.2.2) using a range of available subtype A and subtype B primers. Overlapping PCR products covering the full genome were subsequently generated by BIO-X-ACT using 5µl of cDNA as template. (3.3.1). Products were analysed by agarose gel electrophoresis (3.4) and sequencing (3.5). Sequences were compiled with the aid of a previously known partial subtype B sequence.

4.2.2 Developing an efficient system to readily obtain full length subtype B sequence.

The newly determined subtype B vaccine sequence was used as a base for the design of a multiplex of primers highly specific for an efficient subtype B protocol. In some areas functional subtype A or C primers were maintained. Primers and templates used for each reaction are shown in Table 1. Viral RNA was extracted (3.2.1) and reverse transcribed (3.2.2) into four overlapping sections (RT 1-4 table 1). cDNA generated in reactions RT 1, 2 and 3 was used to amplify eight overlapping sections covering positions 51 to 13,175 of the AMPV-B genome (PCRs 1-8 table 1). BIO-X-ACT enzyme was used (3.3.1) with 5µl of cDNA template and a two minute extension at step 5. RT 1 and RT 4 were then used to generate partial leader and trailer regions of the genome using the Taq PCR protocol (3.3.2) (PCRs 9 and 10, table 1). PCR products were analysed using agar gel electrophoresis on a 1.5% gel for PCRs 1 to 8 and on a 2% gel for PCRs 9 and 10 (3.4). Products were visualised using a UV transilluminator, then prepared for sequencing and analysed as described (3.5). Primers for sequencing are listed in table 1 (SEQ 1-28). To determine the precise sequence of the 3’ and 5’ extremities, RNA was circularised followed by PCRs across the ligated junction as described (4.2.3).

Table 1. Details of primers and templates used in RT-PCR and sequencing reactions. Genome position denotes the binding site at the 5' end of the primer.

Reaction	Primer Name	Sequence 5' to 3'	Template	Genome position
RT 1	Ac le A tr 15 + [a]	acgagaaaaaaacgc	RNA	1
RT 2	M2 Start + [b]	gatgtctaggcgaaatccc	"	4585
RT 3	L2 + [b]	gaaagggaactaagtgtagg	"	8904
RT 4	B trail less 300 + [b]	gtgggctttgtgcaaag	"	13180
PCR 1	N8 + [b]	gtcaaaaatgtctcttgaaagtattagactcagtg	RT 1	51
"	P stop ext– [b]	gacttgtcccatttttttcataactacagatcaag	"	2080
PCR 2	NAB 2 +	ctagatccctcaaagagagcaacaag	"	1110
"	B2680 –	catgaccagaatcaacccctgc	"	2710
PCR 3	MAB 1 +	ggacaacaaccctgcaaaactgac	"	2194
"	FAB 4 –	ctcaactgatgtagcccatgttgc	"	4537
PCR 4	FAB 3 +	ctaatgacttactggacatagaggttaagag	"	4372
"	GAB 3 –	gtagctccctgacaaattggtcctg	"	6427
PCR 5	GAB 1 +	ggcttgacgctcactagcactattg	RT 2	6126
"	B7840 –	catctctgcagcattggacatatcg	"	8410
PCR 6	GAB 4 +	gctgattgagtggtgctgtactag	"	7229
"	LAB 4 –	ccccacacttaattccctttcttttcc	"	9016
PCR 7	LAB 3 +	cgtgtactagagttttacttgaaggatgc	"	8905
"	LAB 9 -	caagttaatgtcctcatttccaaatctctcac	"	11249
PCR 8	LAB 8 +	gtagaccgatggagtttccttcatcag	RT 3	11162
"	L44 –	ctctgaagagttaaggttgtctagcaatttcacc	"	13175
PCR 9	Ac le A tr 15 + [a]	acgagaaaaaaacgc	RT 1	1
"	B lead plus 380 – [b]	gtttagcacctctcgctttc	"	375
PCR 10	B trail less 300 + [b]	gtgggctttgtgcaaag	RT 4	13180
"	C trail 15 – [c]	acggcaaaaaaaccg	"	13509
SEQ 1	N8 + [b]	gtcaaaaatgtctcttgaaagtattagactcagtg	PCR 1	51
SEQ 2	NAB 1 +	tcaaatacccaagaaccaaaagccgtc	"	478
SEQ 3	NAB 2 +	ctagatccctcaaagagagcaacaag	PCR 2	1110
SEQ 4	PAB 1 +	ccgaccctgacgaagataatgatg	"	1649
SEQ 5	MAB 1 +	ggacaacaaccctgcaaaactgac	PCR 3	2194
SEQ 6	MAB 3 +	gagagcttagggaaaatatgcaaaacatgg	"	2816
SEQ 7	FAB 1 +	gctaaaacaataagattagaaggggaggtg	"	3315
SEQ 8	FAB 2 +	atgactatgtgttctgtgatactgcagc	"	3935
SEQ 9	FAB 3 +	ctaatgacttactggacatagaggttaagag	PCR 4	4372
SEQ 10	M2 AB 1 +	gaatccagcaaatctcataaacagtctcaag	"	5004
SEQ 11	SH AB 1 +	cagagctgagcacaactacagc	"	5584
SEQ 12	GAB 1 +	ggcttgacgctcactagcactattg	"	6126
SEQ 13	GAB 2 +	gctgtactgggtggtgttgattg	PCR 5	6378
SEQ 14	G13 + B	caatcctagtcaatcgggaacc	"	7088
SEQ 15	G3 –	actagtacagcaccactc	"	7236
SEQ 16	GAB 4 +	gctgattgagtggtgctgtactag	"	7229
SEQ 17	LAB 1 +	ctggaagtgtcacagaccagtgc	PCR 6	7846
SEQ 18	LAB 2 +	gatatgtccaatgctgcagagatg	"	8407
SEQ 19	LAB 3 +	cgtgtactagagttttacttgaaggatgc	PCR 7	8905
SEQ 20	LAB 5 +	ccatggaggcaatatctctccttgatg	"	9518
SEQ 21	LAB 6 +	ggagaccctgttgttgtgtataggag	"	10123
SEQ 22	LAB 7 +	cattgatagagcagttcatatgatgttgctc	"	10614
SEQ 23	LAB 8 +	gtagaccgatggagtttccttcatcag	PCR 8	11162
SEQ 24	LAB 10 +	gacattgtagaagacccaattgacaggc	"	11782
SEQ 25	LAB 11 +	cataactttgctgccttggcacc	"	12309
SEQ 26	LAB 12 +	cacagctccttgctatggagagg	"	12933
SEQ 27	B lead plus 380 neg	gtttagcacctctcgctttc	PCR 9	375
SEQ 28	B trail less 300 +	gtgggctttgtgcaaag	PCR 10	13180

[a] Based on sequence of AMPV-A and C (Genebank acc: AY640317 & AY579780.1 respectively)
[b] Based on sequence of AMPV-A
[c] Based on sequence of AMPV-C

4.2.3 RNA circularisation to determine 3' leader and 5' trailer sequences.

An RNA circularisation method was performed to determine the 3' and 5' genome extremities (figure 2). Different volumes of RNA in ligations and RT reactions were used and these are shown in table 2 and all primers are shown in table 3.

RNA was extracted (3.2.1) and then circularised overnight using T4 RNA Ligase (3.6). Ligated material was then used as template in RT reactions (3.2.2) using the L gene specific forward primer L19+ to generate cDNA across the ligated junction. GoTaq PCR's (3.3.2) were performed using the same forward L gene primer (L19+) and a reverse primer specific for the N gene (N4 -). For further amplification nested GoTaq PCRs were then used with primers L6+A and N2 -. In these PCRs 0.5µl of the initial PCR template was used. PCR products of the expected size (357nt) were sequenced with primers L6+A and N2- (3.5).

Table 2. Volumes of RNA used for determining genome extremities.

Ligation	Volume (µl) of RNA	Volume (µl) of ligation in RT-PCR
1	10	5
2	5	3
3	10	3

Table 3. Sequence of primers used to determine genome extremities. Genome position denotes the binding site at the 5' end of the primer.

Reaction	Primer Name	Sequence 5' to 3'	Genome position
RT	L19+	gaagtggttaaatcacgttctg	13,088
PCR	L19+	gaagtggttaaatcacgttctg	13,088
	N4-	cttcaacagttgaggccaac	560
Nested PCR	L6+A	ctcaacacataattatcacctg	13,271
	N2-	gcatgcctacctctgctg	240

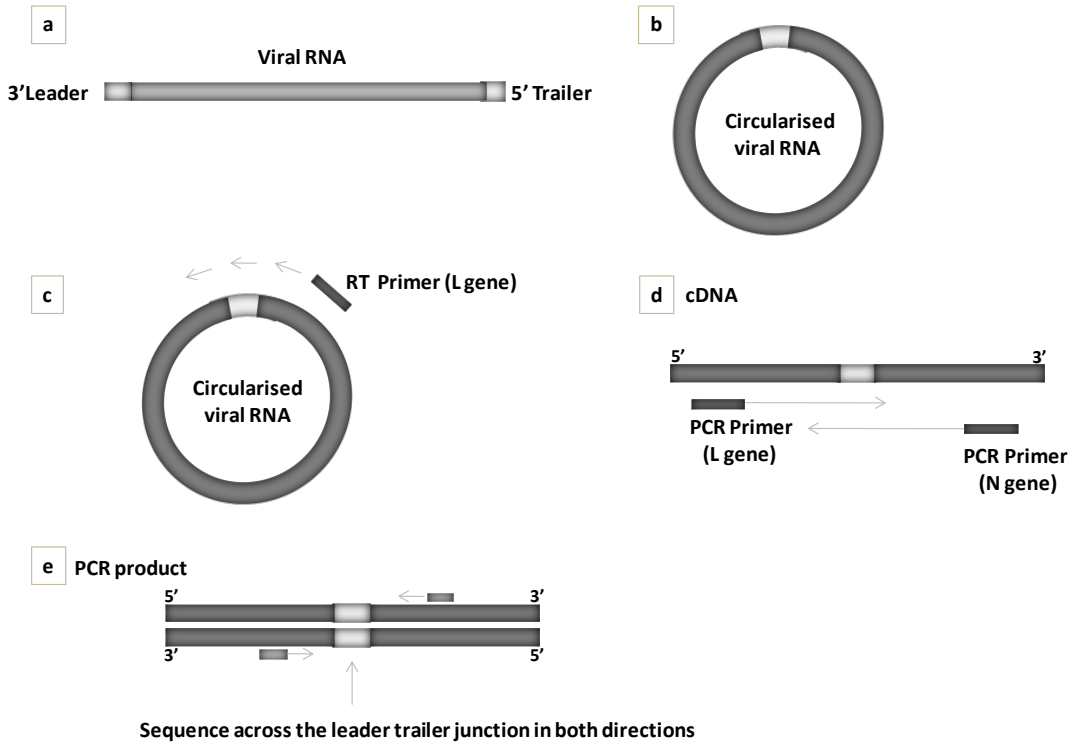


Figure 2. RNA circularisation and RT-PCR. Extracted RNA (a) was circularised with T4 RNA ligase (b), followed by RT across the ligated junction using an L gene specific forward primer (c). Nested PCRs were performed with L gene specific forward and N gene specific reverse primers (d). Generated products were sequenced in both directions (e).

4.2.4 Chicken and turkey-derived AMPV comparison study.

FL AMPV-A and B sequences of field and vaccine origin derived from chickens and turkeys were determined using a subtype A RT-PCR system and the subtype B RT-PCR system described in section 4.2.2. Details of the selected viruses are shown in table 4. Each virus was given a reference code such as CA:1 (table 1). The first letter corresponds to the host from where the virus was isolated, C (chicken) or T (turkey), the second letter corresponds to the AMPV subgroup, and the number corresponds to the number of viruses in this category. The vaccines used were reconstituted with 2ml of sterile de-ionised water before use. Field strains had previously undergone a small number of passages in TOC, before storage at -20^{o}C, with the exception of TA:3 which had been adapted to Vero cells. Sequences of subtype A AMPVs TA: 3, 4, 5, 6 and 7 had been determined prior to this study.

Isolate TA: 5 was the progenitor virus of commercial vaccines TA: 6 and TA: 7. Isolate TA: 1 was the progenitor virus for vaccine TA: 8.

The full genome sequences of all the listed viruses were aligned using Bioedit Sequence Alignment Editor (version 7.0.9.0). Each sequence was then analysed using Generunner (version 3.05) to predict putative ORFs. Amino acid sequences of each ORF of each virus were then also aligned using Bioedit software.

Table 4. AMPV field and vaccine isolates used in molecular comparisons

Ref	Species	Subtype	Field strain (F) or Vaccine (V) strain	Vaccine company or country of isolation
C A :1	Chicken	A	F	UK
T A : 1	Turkey	A	F	UK
T A : 2	Turkey	A	F	Italy
T A : 3	Turkey	A	F	Germany
T A : 4	Turkey	A	F	UK
T A : 5	Turkey	A	F	UK
T A : 6	Turkey	A	V	Fort Dodge
T A : 7	Turkey	A	V	Intervet
T A : 8	Turkey	A	V	Pitman-Moore
C B : 1	Chicken	B	F	France
C B : 2	Chicken	B	F	Holland
C B : 3	Chicken	B	F	Spain
C B : 4	Chicken	B	V	Merial
C B : 5	Chicken	B	V	Intervet
T B : 1	Turkey	B	F	Italy
T B : 2	Turkey	B	F	Italy
T B : 3	Turkey	B	F	Italy
T B : 4	Turkey	B	F	France
T B : 5	Turkey	B	V	Merial

4.2.5 Passage of turkey-derived AMPV in chicken TOC.

Subtype A (TA:7) and B (TB:1-5) turkey-derived AMPV isolates were passaged ten times in chicken TOC in an attempt to generate species specific mutations. In brief embryonated SPF chicken eggs (Lohmann Animal Health,Cuxhaven, Germany) were used to prepare TOCs from 19 day old embryos as previously reported (59).

Viable rings were inoculated with 100µl of each of the above mentioned viruses (each adjusted to 3.5 Log_{10} TCID50 per ml) and incubated at 37^{o}C. TOC rings were visualised daily for cillial activity and at cilliostasis, or after seven days incubation, rings were freeze-thawed and centrifuged. Supernatant was collected and used to inoculate new TOCs (Figure 3).

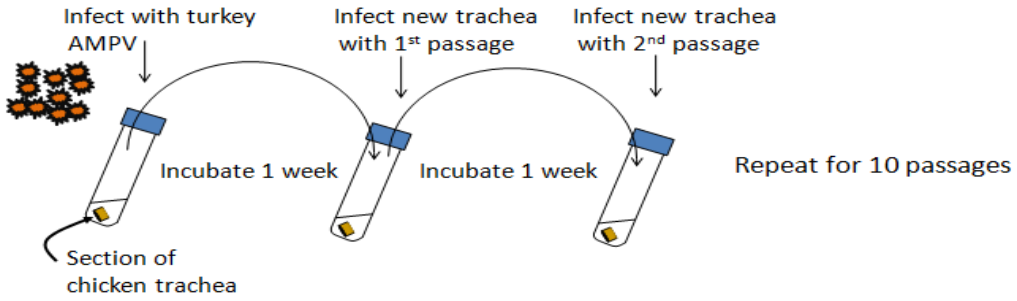


Figure 3. Chicken TOC passage of turkey-derived AMPV isolates.

4.3 Results

4.3.1 Full length subtype B RT-PCR protocol.

PCR 1 through to 8 generated products of approximately 2kbp, 1.6kbp, 2.3kbp, 2.1kbp, 2.2kbp, 1.9kbp, 2.3kbp, and 2kbp (figure 4). PCR 9 and 10 generated products of 395 bp and 334 bp respectively (figure 5).

In general these products generated clear sequencing data. However on occasions when data was mixed or impossible to determine, the PCR and / or sequencing protocol was repeated.

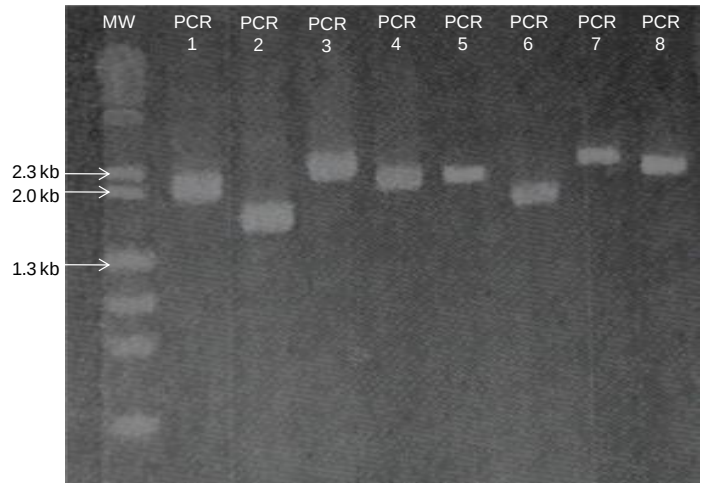


Figure 4. Products amplified in PCRs 1 to 8 visualised on a 1.5% agarose gel.

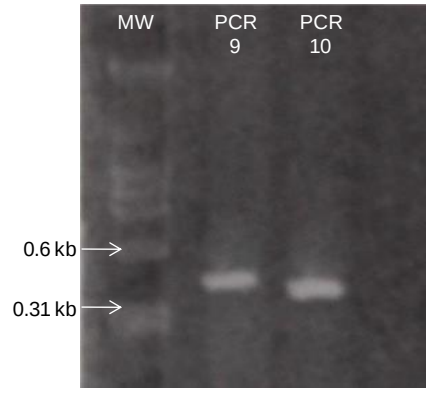


Figure 5. Products amplified in PCRs 9 and 10 visualised on a 2% agarose gel.

4.3.2 3’ and 5’ genome extremities.

The following data were obtained from a subtype A UK field strain (TA:1). All sequences are shown 5’ to 3’ DNA for ease of explanation. Sequence at the 3’ extremity of the genome, generated from ligation 1 using reverse primer N2-, was tentatively deduced as ACGAGAAAAAAACGC (A, figure 6). A 3’ sequence of GAAAAAAACGC was generated from both ligations 2 and 3 (B and D, figure 6) using reverse primer N2- . However, both these sequences lacked the ACGA bases seen at the 3’ end in ligation 1 (A, figure 6).

5’ sequences generated from ligations 2 and 3 were found to be CGGTTTTTTTCTCGT or CGGTTTTTTTTTCGT respectively (C and D figure 6). The 5’ sequence produced from ligation 1 (A, figure 6) was unclear. Sequence produced from ligation 2 was complimentary to the start sequence produced in ligation 1 (figure 7). This is consistent with the sequence reported previously for AMPV-A. It would also allow the formation of the previously described essential pan handle structure of the genome ends.

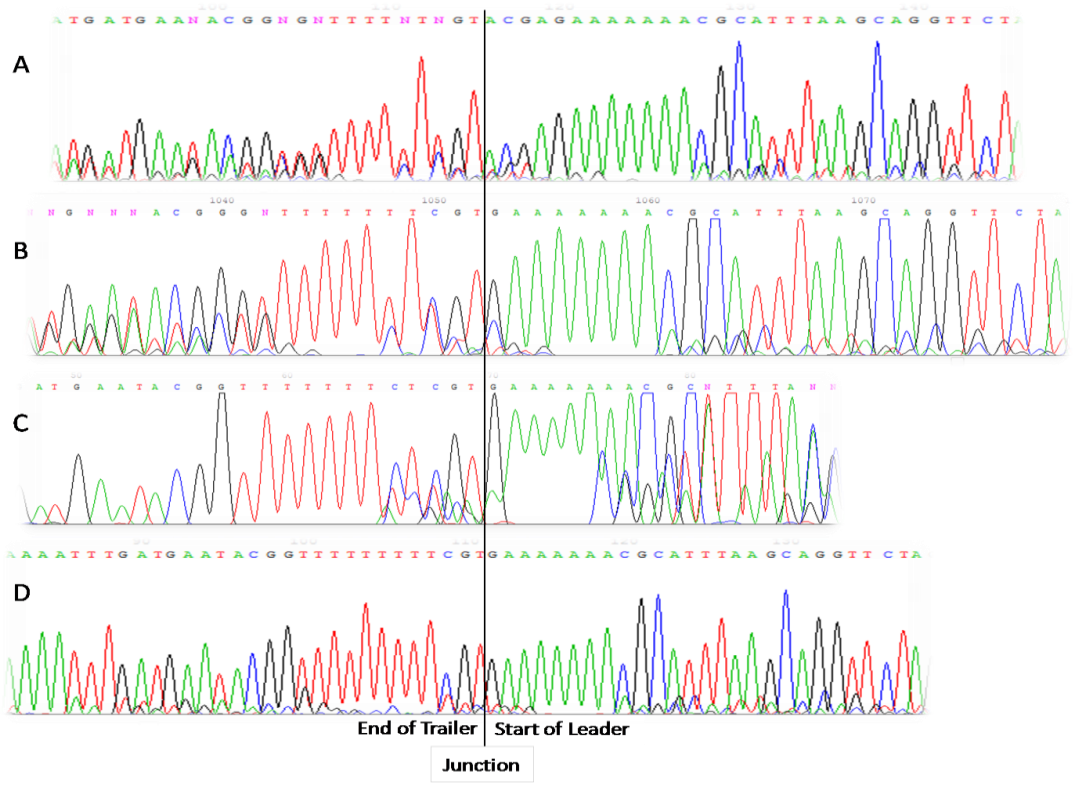


Figure 6. Chromatograms of sequence generated from circularized genomic RNA in ligation 1 (A), 2 (B, C) and 3 (D). The line represents the proposed junction between the end and start of the genome. Complementary genomic extremities consistent with those reported previously for AMPV-A can be obtained using the leader start sequence of ligation 1 (A) and the trailer end sequence of ligation 2 (C).

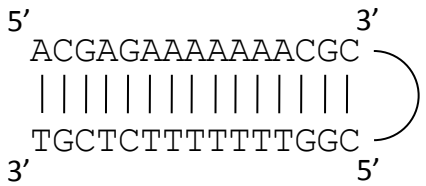


Figure 7. Complimentarity of deduced genomic ends for an AMPV-A isolate (TA:1).

A large majority of sequence data was unreadable using this method for the other subtype A and B isolates used in this study despite varying the ligation conditions, and using different primers and primer combinations. This method alone did not provide a fast efficient means of attaining sequence of the 3' and 5' extremities. Thus, these regions were not taken into account in the comparison study.

4.3.3 Comparisons of chicken and turkey-derived subtype A AMPV genomes and deduced amino acid sequences.

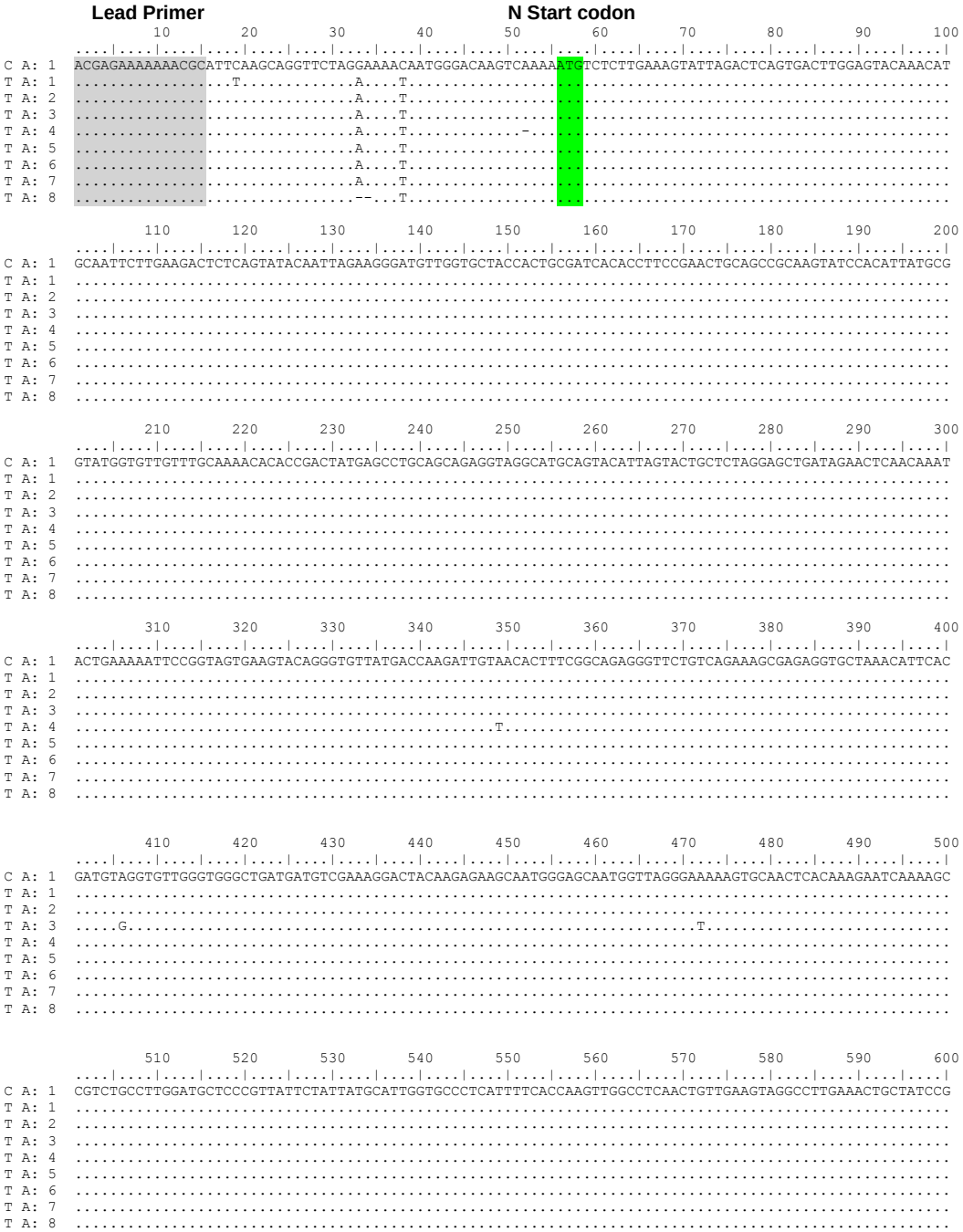
(i) Nucleotide sequence comparison.

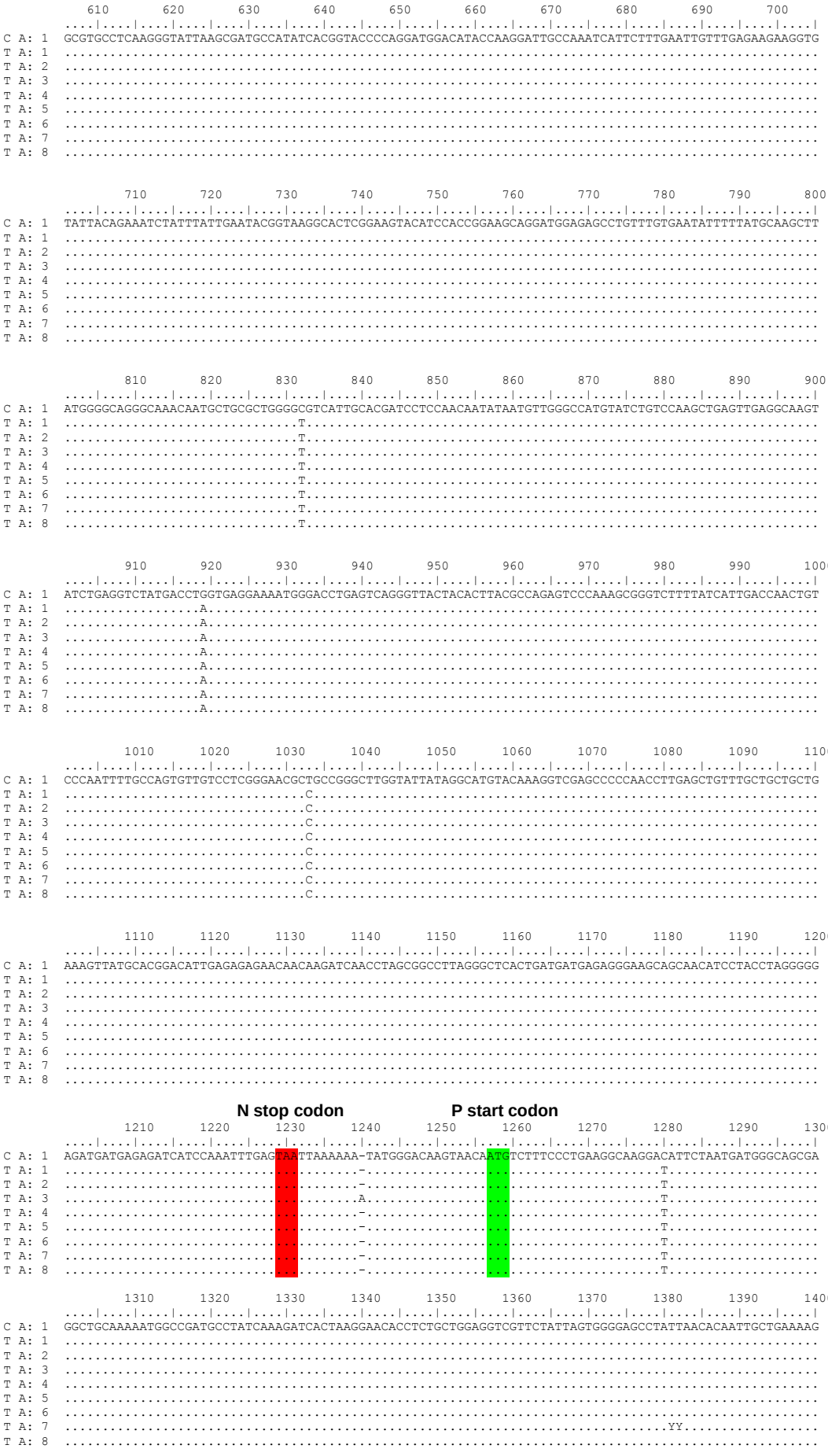
Only one chicken-derived subtype A isolate, CA:1, could be sourced for the comparison study of subtype A AMPVs . This isolate possessed 34 nt that were different from all turkey-derived AMPV-A sequences. Eleven of these were non silent and are described in the following section (4.3.3 ii). CA:1 also differed from all turkey AMPV sequences at two positions in the leader region before the translation start of the N ORF, and at one position between the SH ORF translation stop and the G ORF translation start codon. One extra nt was observed between the M2 ORF translation stop and the SH ORF translation start codons.

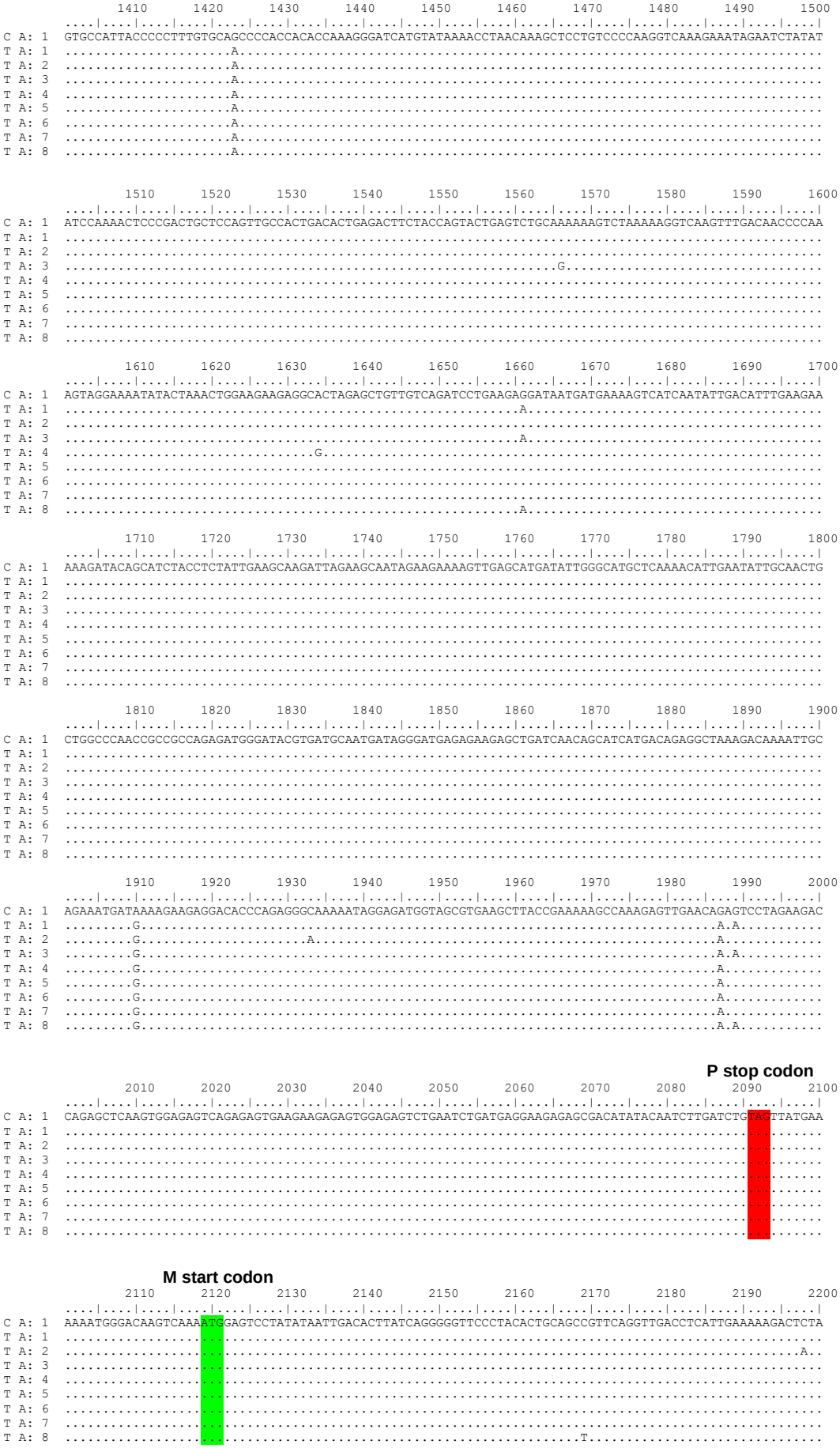
The nt sequences of turkey AMPV-A isolates were highly conserved and although some random individual differences were seen no host specific motifs could be identified. Nucleotide sequences were 100% conserved in the region between the translation stop and translation start codons of the P and M ORFs and F and M2 ORFs, and in the determined trailer sequence. Individual differences including base deletions and insertions were observed in all other non-coding regions.

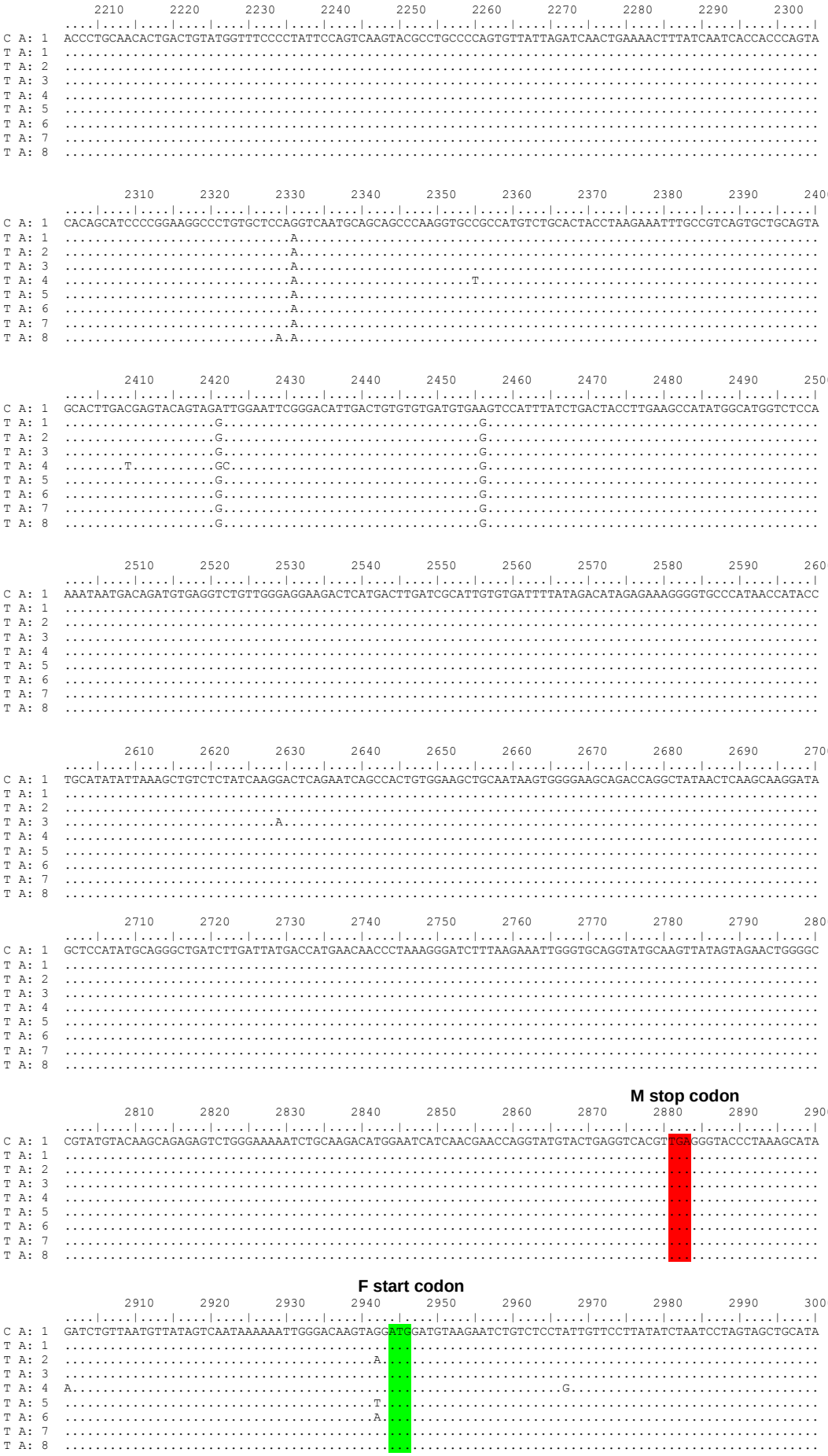
Eight nt differences were noted between the turkey field strain TA:1 and its vaccine derivative TA:8. Six of these resulted in aa codon differences (see 4.3.3 ii).

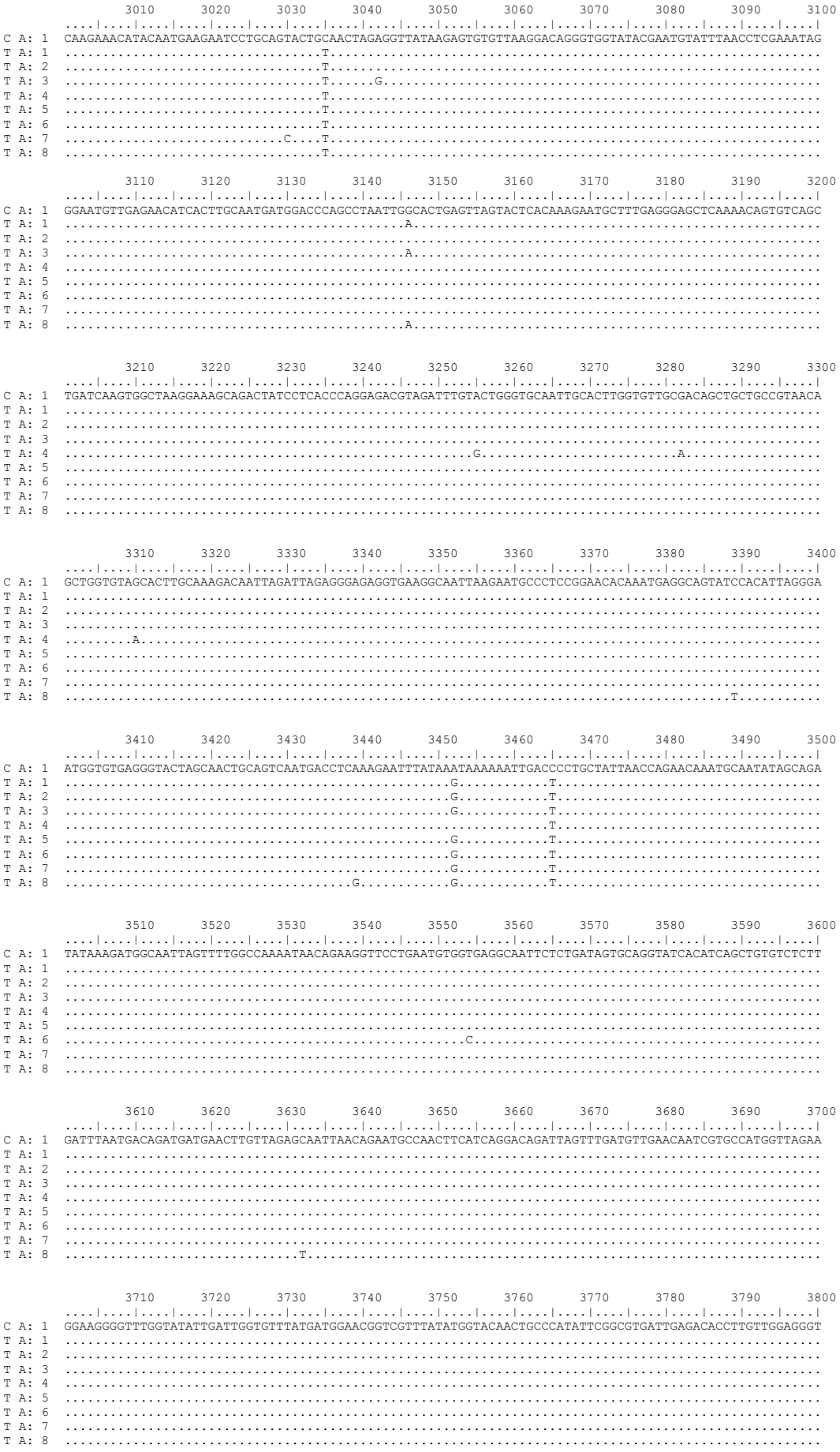
The full nt sequence alignments of subtype A-AMPV's are shown in figure 8. ORF translation start and stop codons are highlighted in green and red respectively. 3' leader and 5' trailer extremities were not taken into account in the comparison, therefore in the given nt alignments the sequence of the extremities is that of the primers used in PCR amplifications. These are highlighted in grey. Ambiguity code Y represents nt C or T.

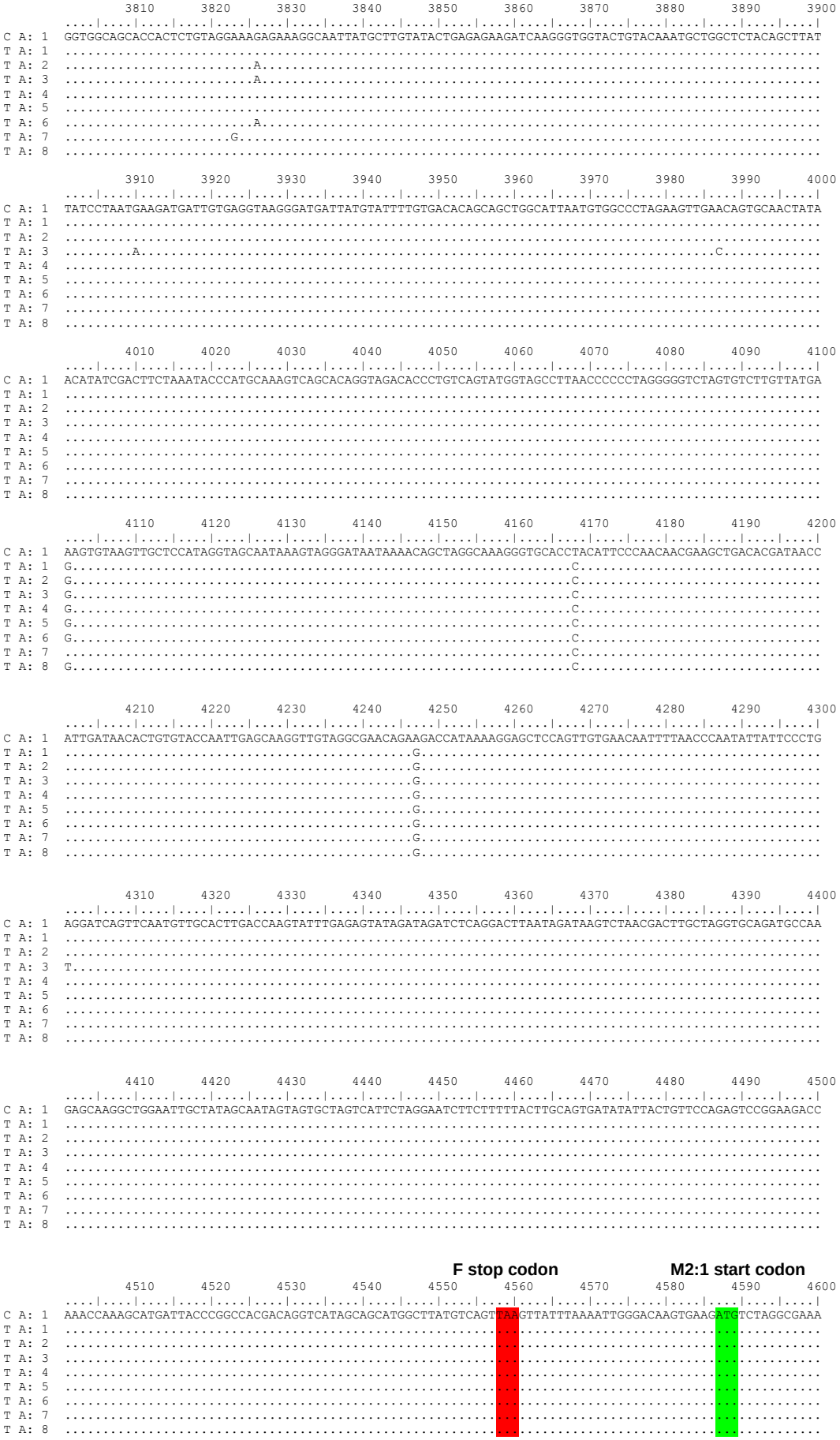


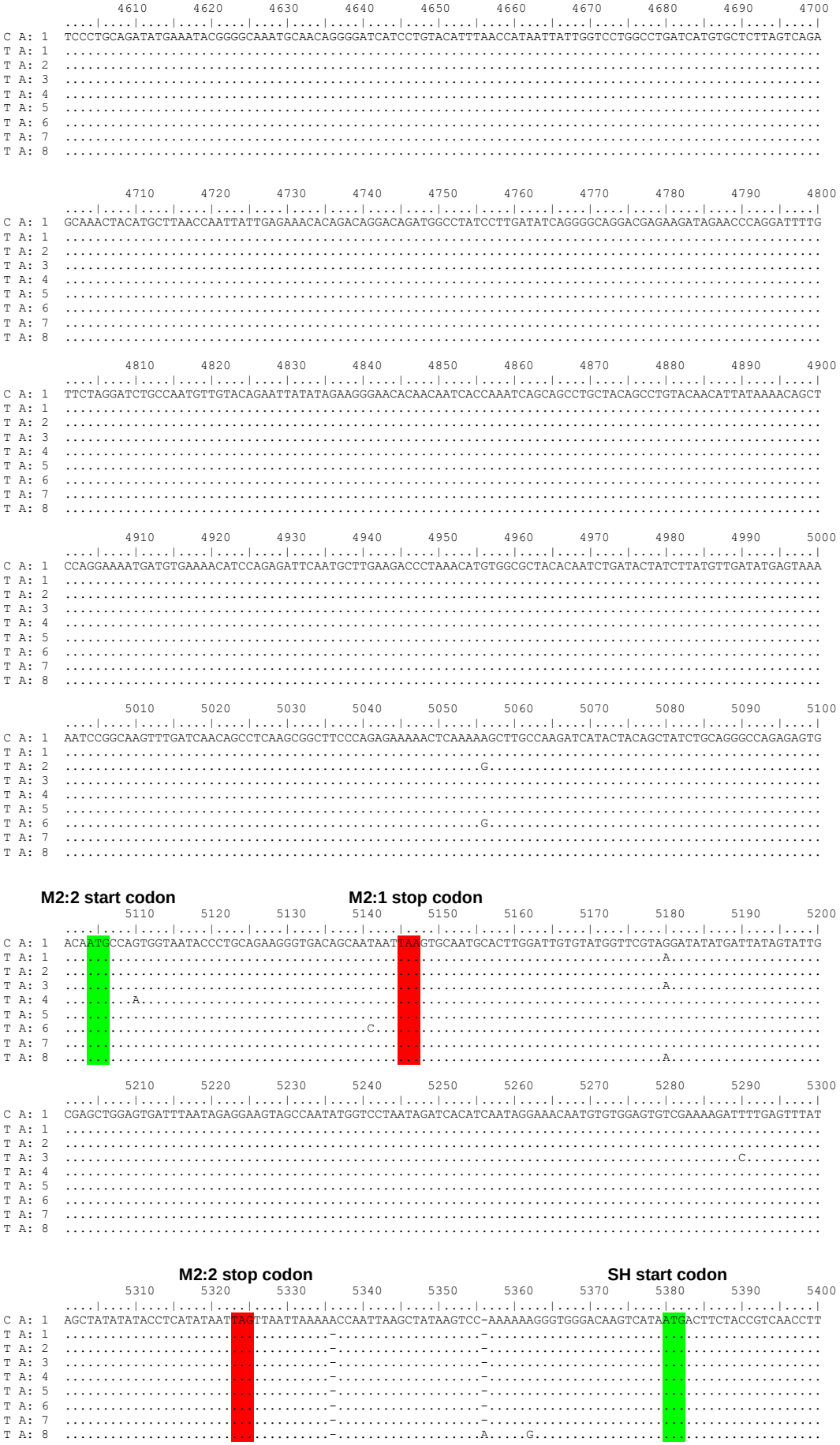

N stop codon
P start codon

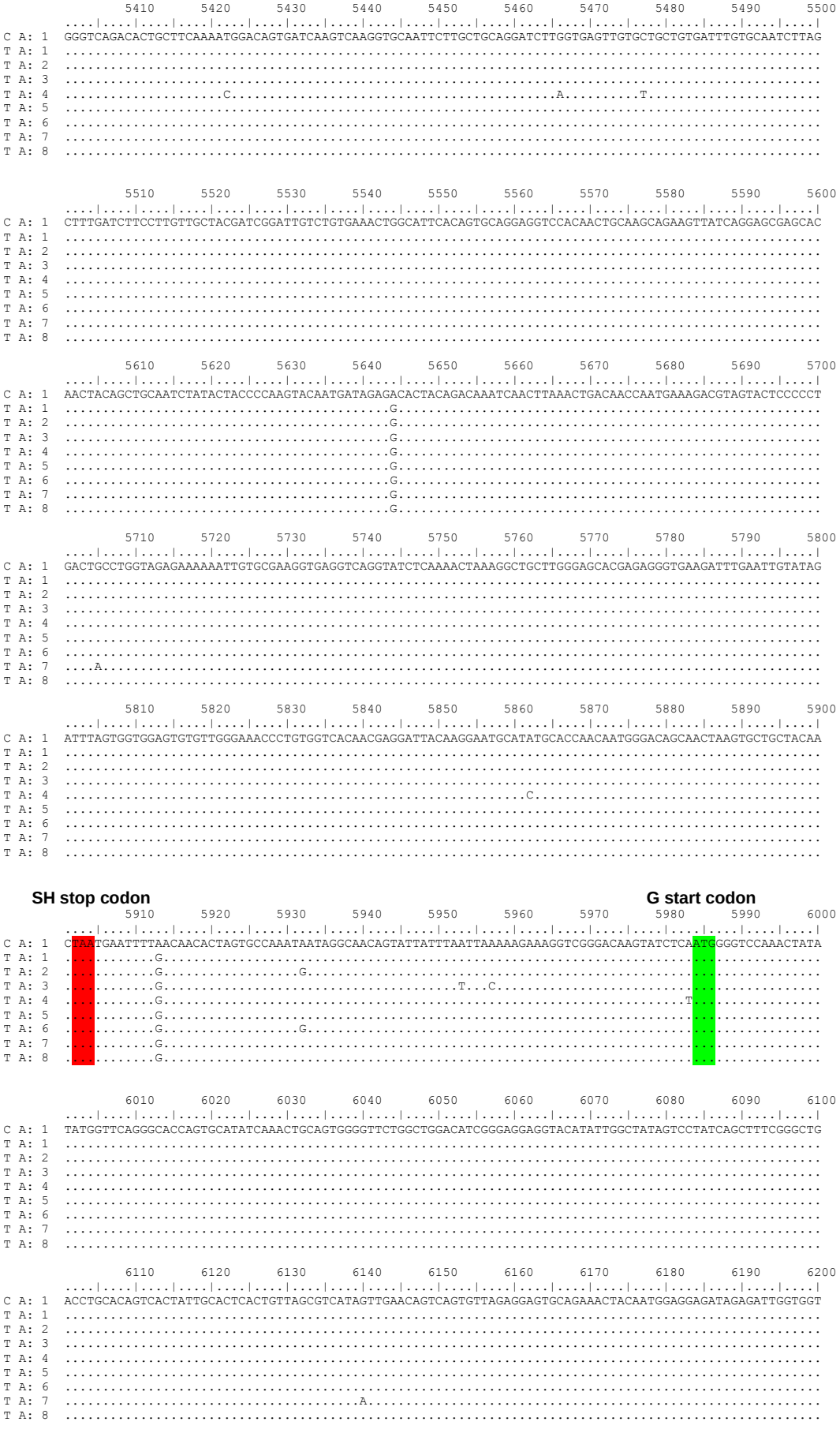

P stop codon
M start codon

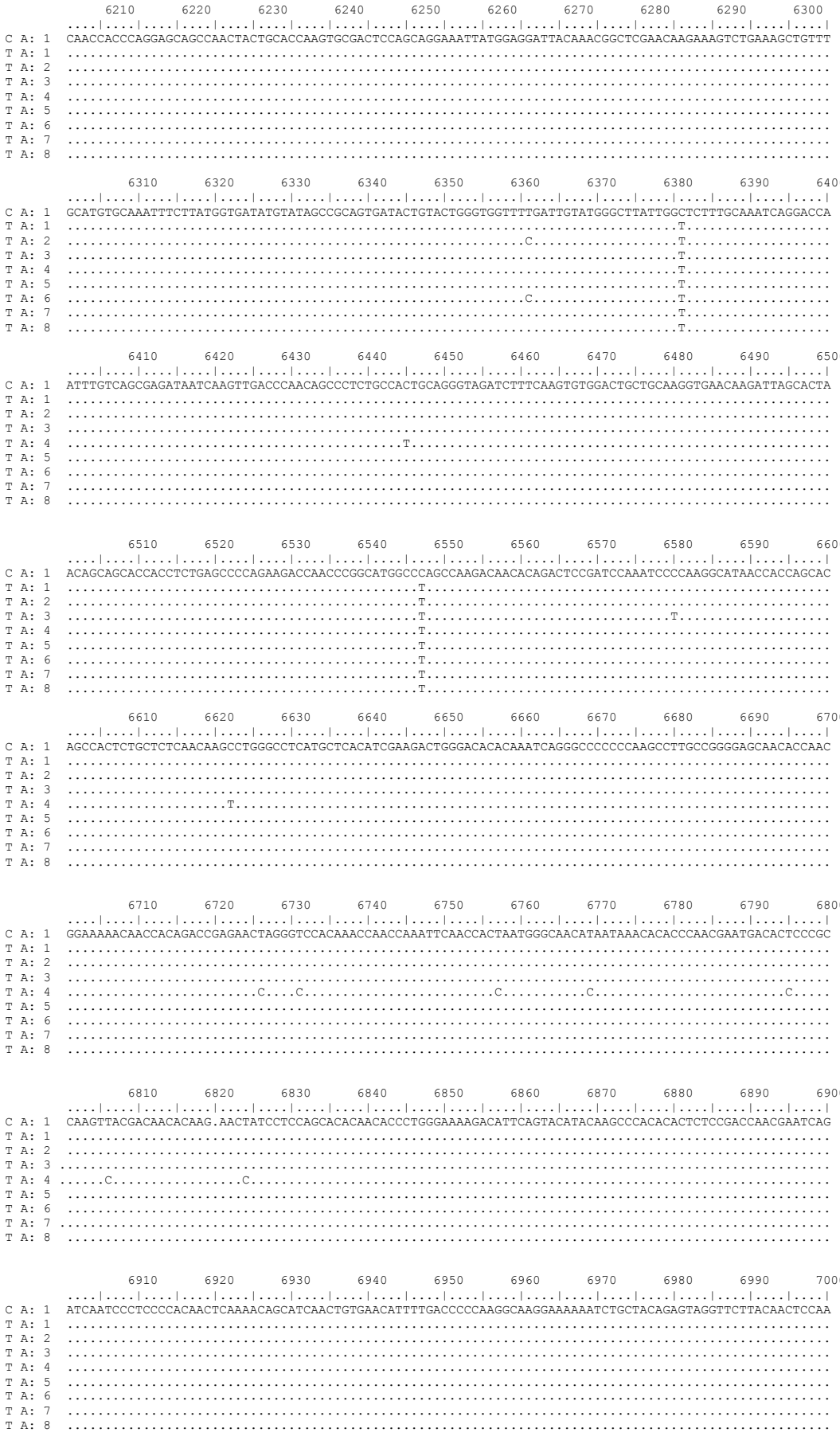

M stop codon
F start codon

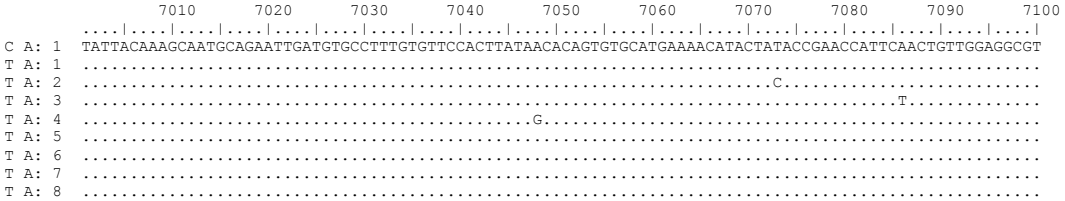

3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
C A: 1 CAAGAAACATACAATGAAGAATCCTGCAGTACTGCAACTAGAGGTTATAAGAGTGTGTTAAGGACAGGGTGGTATACGAATGTATTTAACCTCGAAATAG
T A: 1 .....T.....
T A: 2 .....T.....
T A: 3 .....T.....G.....
T A: 4 .....T.....
T A: 5 .....T.....
T A: 6 .....T.....
T A: 7 .....C....T.....
T A: 8 .....T.....
3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
C A: 1 GGAATGTTGAGAACATCACTTGCAATGATGGACCCAGCCTAATTGGCACTGAGTTAGTACTCACAAAGAATGCTTTGAGGGAGCTCAAAACAGTGTCAGC
T A: 1 .....A.....
T A: 2 .....
T A: 3 .....A.....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....A.....
3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
C A: 1 TGATCAAGTGGCTAAGGAAAGCAGACTATCCTCACCCAGGAGACGTAGATTTGTACTGGGTGCAATTGCACTTGGTGTTGCGACAGCTGCTGCCGTAACA
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....G.....A.....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
C A: 1 GCTGGTGTAGCACTTGCAAAGACAATTAGATTAGAGGGAGAGGTGAAGGCAATTAAGAATGCCCTCCGGAACACAAATGAGGCAGTATCCACATTAGGGA
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....A.....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....T.....
3410 3420 3430 3440 3450 3460 3470 3480 3490 3500
C A: 1 ATGGTGTGAGGGTACTAGCAACTGCAGTCAATGACCTCAAAGAATTTATAAATAAAAAATTGACCCCTGCTATTAACCAGAACAAATGCAATATAGCAGA
T A: 1 .....G.....T.....
T A: 2 .....G.....T.....
T A: 3 .....G.....T.....
T A: 4 .....T.....
T A: 5 .....G.....T.....
T A: 6 .....G.....T.....
T A: 7 .....G.....T.....
T A: 8 .....G.....G.....T.....
3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
C A: 1 TATAAAGATGGCAATTAGTTTTGGCCAAAATAACAGAAGGTTCCTGAATGTGGTGAGGCAATTCTCTGATAGTGCAGGTATCACATCAGCTGTGTCTCTT
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....C.....
T A: 7 .....
T A: 8 .....
3610 3620 3630 3640 3650 3660 3670 3680 3690 3700
C A: 1 GATTTAATGACAGATGATGAACTTGTTAGAGCAATTAACAGAATGCCAACTTCATCAGGACAGATTAGTTTGATGTTGAACAATCGTGCCATGGTTAGAA
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....T.....
3710 3720 3730 3740 3750 3760 3770 3780 3790 3800
C A: 1 GGAAGGGGTTTGGTATATTGATTGGTGTTTATGATGGAACGGTCGTTTATATGGTACAACTGCCCATATTCGGCGTGATTGAGACACCTTGTTGGAGGGT
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....


F stop codon
M2:1 start codon

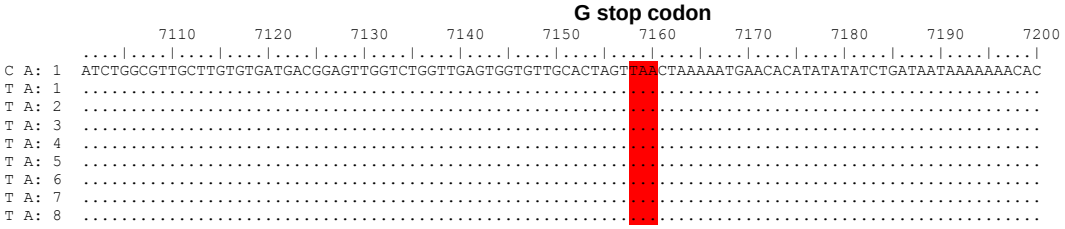

M2:2 start codon
M2:1 stop codon
M2:2 stop codon
SH start codon


SH stop codon
G start codon

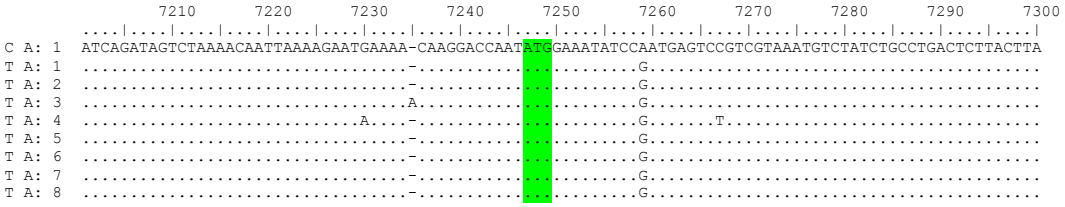

6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
C A: 1 CAACCACCCAGGAGCAGCCAACTACTGCACCAAGTGCGACTCCAGCAGGAAATTATGGAGGATTACAAACGGCTCGAACAAGAAAGTCTGAAAGCTGTTT
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6310 6320 6330 6340 6350 6360 6370 6380 6390 6400
C A: 1 GCATGTGCAAATTTCTTATGGTGATATGTATAGCCGCAGTGATACTGTACTGGGTGGTTTTGATTGTATGGGCTTATTGGCTCTTTGCAAATCAGGACCA
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6410 6420 6430 6440 6450 6460 6470 6480 6490 6500
C A: 1 ATTTGTCAGCGAGATAATCAAGTTGACCCAACAGCCCTCTGCCACTGCAGGGTAGATCTTTCAAGTGTGGACTGCTGCAAGGTGAACAAGATTAGCACTA
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6510 6520 6530 6540 6550 6560 6570 6580 6590 6600
C A: 1 ACAGCAGCACCACCTCTGAGCCCCAGAAGACCAACCCGGCATGGCCCAGCCAAGACAACACAGACTCCGATCCAAATCCCCAAGGCATAACCACCAGCAC
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700
C A: 1 AGCCACTCTGCTCTCAACAAGCCTGGGCCTCATGCTCACATCGAAGACTGGGACACACAAATCAGGGCCCCCCCAAGCCTTGCCGGGGAGCAACACCAAC
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6710 6720 6730 6740 6750 6760 6770 6780 6790 6800
C A: 1 GGAAAAACAACCACAGACCGAGAACTAGGGTCCACAAACCAACCAAATTCAACCACTAATGGGCAACATAATAAACACACCCAACGAATGACACTCCCGC
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6810 6820 6830 6840 6850 6860 6870 6880 6890 6900
C A: 1 CAAGTTACGACAACACAAG.AACTATCCTCCAGCACACAACACCCTGGGAAAAGACATTCAGTACATACAAGCCCACACACTCTCCGACCAACGAATCAG
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8
6910 6920 6930 6940 6950 6960 6970 6980 6990 7000
C A: 1 ATCAATCCCTCCCCACAACTCAAAACAGCATCAACTGTGAACATTTTGACCCCCAAGGCAAGGAAAAAATCTGCTACAGAGTAGGTTCTTACAACTCCAA
T A: 1
T A: 2
T A: 3
T A: 4
T A: 5
T A: 6
T A: 7
T A: 8

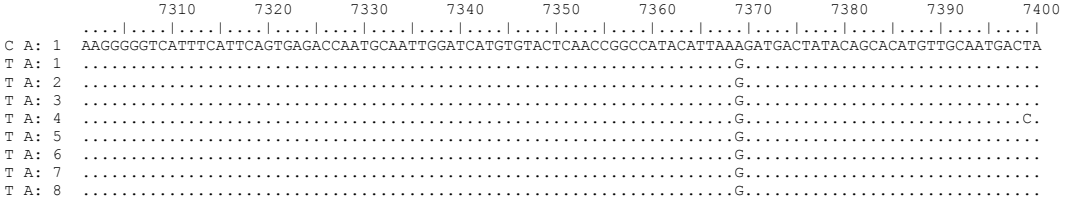


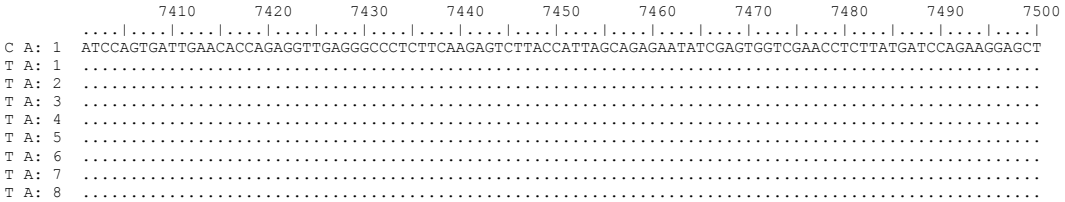
G stop codon

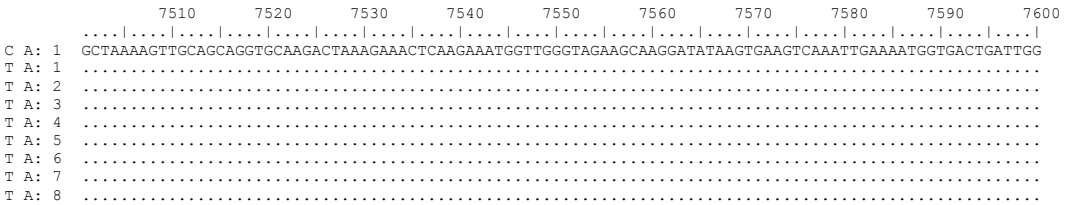


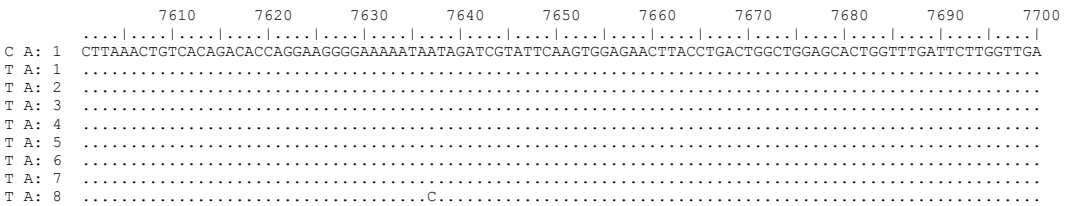
L start codon











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            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  TTTCGGGTGTATAATCATAAGTCGCAAGAGTAGAAGAATATGTTTTTGTACCTACAATCAACTCCTGACTTGGAAGGACCTTGCCTTGAGCAGATTTAAT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                7910      7920      7930      7940      7950      7960      7970      7980      7990      8000
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  GCAAACTTGTGTGTGTGGGTGAGCAATTGCTTAAATAGTGCCCAAGACGGCCTTGGATTAAGGAGCAAATTAGTTGGTGAGTTACTCAACCGATTGTACT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                8010      8020      8030      8040      8050      8060      8070      8080      8090      8100
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  GTGAGACTGATGAGTTATTGAGCATCACTGGCAATGAGGGGTATGGGATTGTAAAGGAATTTGAAGGATTCATTATGAGCGAAATATTGCGGATGACTGA
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                8110      8120      8130      8140      8150      8160      8170      8180      8190      8200
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  ACATGCACAATTCAGTGTAAGGTTCCGGAATACGCTATTGAATGGCATAGCTGATAAAATAGGGAAGATGAGAGCTGCATACCGAAGGCGGACACAGAAC
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ........................A...........................................................................
T A: 8  ....................................................................................................

                8210      8220      8230      8240      8250      8260      8270      8280      8290      8300
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  ACATCTGTTGAGCATCATAGATACCCAGGCGAGCATGAAATGCTGGAAGCAACAGGGAGAGTGCTGAGAATAATTAAGCTTCTGGTGAATAATGAGATTG
T A: 1  ....................................................................................................
T A: 2  ..............................................................C.....................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ..............................................................C.....................................
T A: 6  ..............................................................C.....................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                8310      8320      8330      8340      8350      8360      8370      8380      8390      8400
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CTAATGCTGCAGAGATGTATTTTATATTCCGCATATTCGGACATCCTATGGTAGAAGAAAGGGAGGCTATGGATGCAGTGAGGGATAACAGTGAAACTGC
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                8410      8420      8430      8440      8450      8460      8470      8480      8490      8500
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  AAAAATCTTAAGTCTGGTGGCCCTTACGGAGATGAGGGGGGCCTTCATTCTGAGAGTGATCAAAGGTTTCGTAGGGAACTACAAAAGATGGCCAAAGATA
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                8510      8520      8530      8540      8550      8560      8570      8580      8590      8600
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  AAGAATCCATCAACATTGAGTCGAAGGTGGAAGATGTACATGAATGCAAAAACATACCCAAGCAATCTTGAATTGTGTGTGGAAGACTTCTTGGAGCTCG
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................
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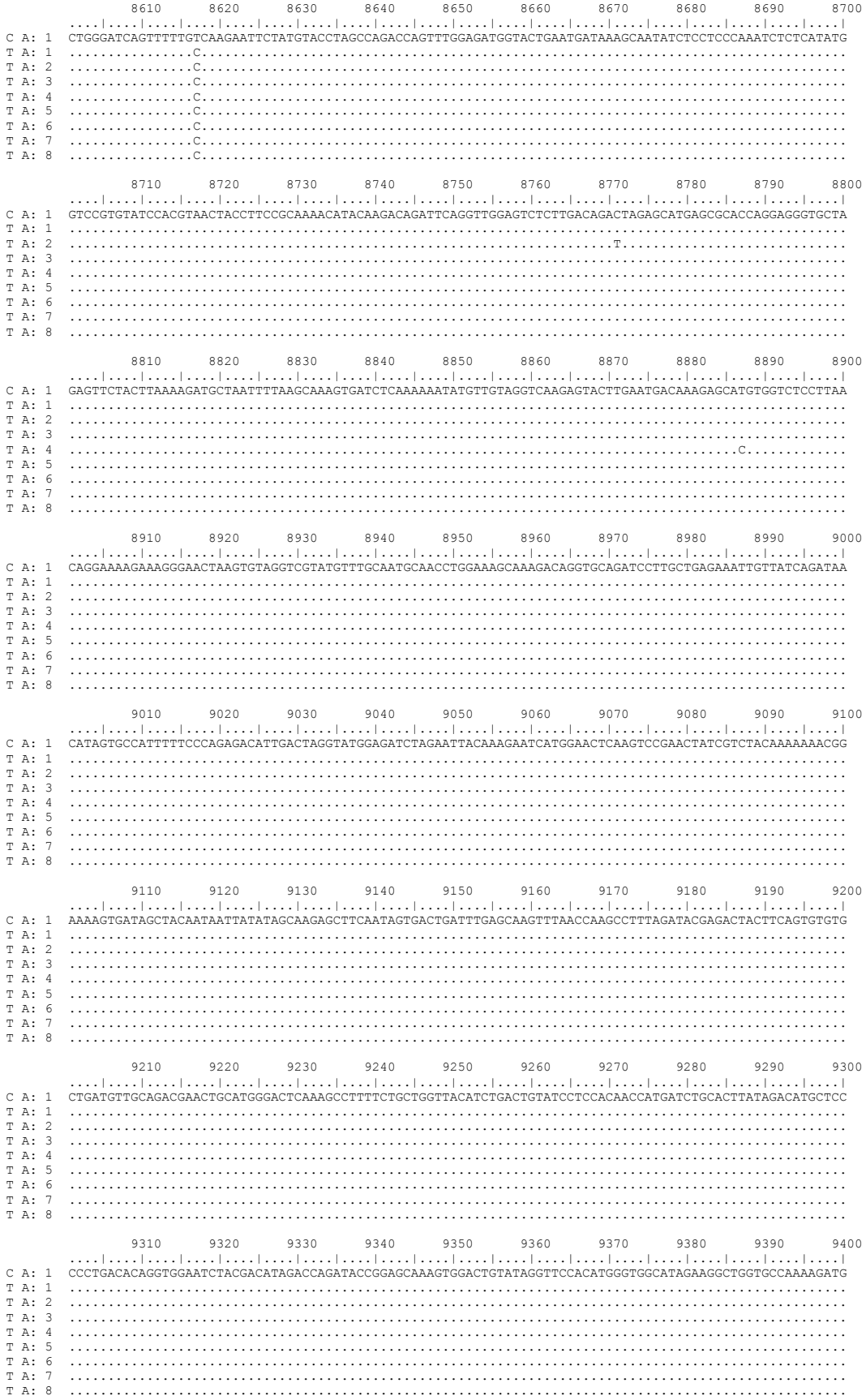
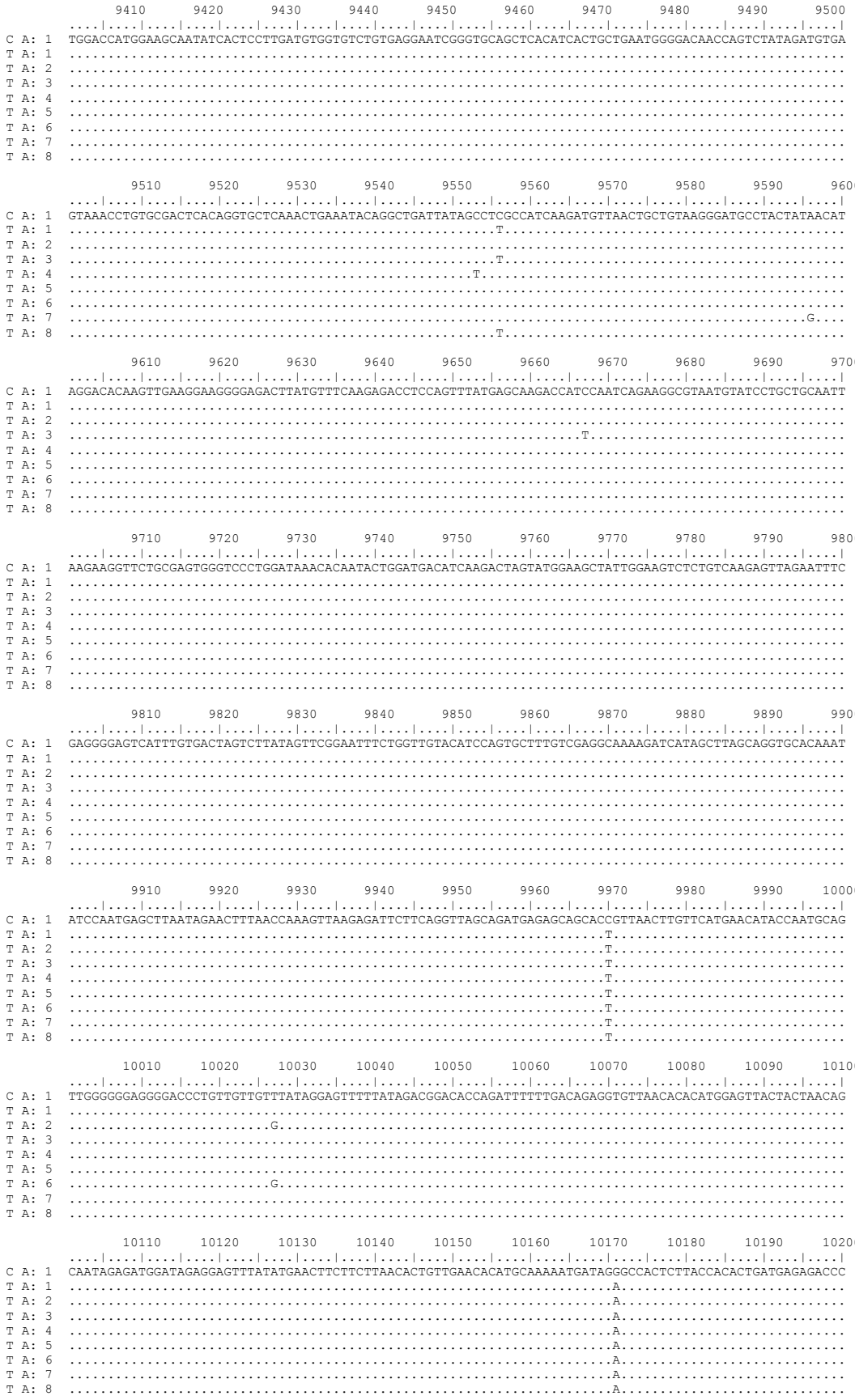

8610 8620 8630 8640 8650 8660 8670 8680 8690 8700
C A: 1 CTGGGATCAGTTTTTGTCAAGAATTCTATGTACCTAGCCAGACCAGTTTGGAGATGGTACTGAATGATAAAGCAATATCTCCTCCCAAATCTCTCATATG
T A: 1 .....C.....
T A: 2 .....C.....
T A: 3 .....C.....
T A: 4 .....C.....
T A: 5 .....C.....
T A: 6 .....C.....
T A: 7 .....C.....
T A: 8 .....C.....
8710 8720 8730 8740 8750 8760 8770 8780 8790 8800
C A: 1 GTCCGTGTATCCACGTAACTACCTTCCGCAAAACATACAAGACAGATTCAGGTTGGAGTCTCTTGACAGACTAGAGCATGAGCGCACCAGGAGGGTGCTA
T A: 1 .....
T A: 2 .....T.....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
8810 8820 8830 8840 8850 8860 8870 8880 8890 8900
C A: 1 GAGTTCTACTTAAAAGATGCTAATTTTAAGCAAAGTGATCTCAAAAAATATGTTGTAGGTCAAGAGTACTTGAATGACAAAGAGCATGTGGTCTCCTTAA
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....C.....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
8910 8920 8930 8940 8950 8960 8970 8980 8990 9000
C A: 1 CAGGAAAAGAAAGGGAACTAAGTGTAGGTCGTATGTTTGCAATGCAACCTGGAAAGCAAAGACAGGTGCAGATCCTTGCTGAGAAATTGTTATCAGATAA
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
9010 9020 9030 9040 9050 9060 9070 9080 9090 9100
C A: 1 CATAGTGCCATTTTTCCCAGAGACATTGACTAGGTATGGAGATCTAGAATTACAAAGAATCATGGAACTCAAGTCCGAACTATCGTCTACAAAAAAACGG
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
9110 9120 9130 9140 9150 9160 9170 9180 9190 9200
C A: 1 AAAAGTGATAGCTACAATAATTATATAGCAAGAGCTTCAATAGTGACTGATTTGAGCAAGTTTAACCAAGCCTTTAGATACGAGACTACTTCAGTGTGTG
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
9210 9220 9230 9240 9250 9260 9270 9280 9290 9300
C A: 1 CTGATGTTGCAGACGAACTGCATGGGACTCAAAGCCTTTTCTGCTGGTTACATCTGACTGTATCCTCCACAACCATGATCTGCACTTATAGACATGCTCC
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
9310 9320 9330 9340 9350 9360 9370 9380 9390 9400
C A: 1 CCCTGACACAGGTGGAATCTACGACATAGACCAGATACCGGAGCAAAGTGGACTGTATAGGTTCCACATGGGTGGCATAGAAGGCTGGTGCCAAAAGATG
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....


9410 9420 9430 9440 9450 9460 9470 9480 9490 9500
C A: 1 TGGACCATGGAAGCAATATCACTCCTTGATGTGGTGTCTGTGAGGAATCGGGTGCAGCTCACATCACTGCTGAATGGGGACAACCAGTCTATAGATGTGA
T A: 1 T
T A: 2 T
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 T
T A: 7 T
T A: 8 T
9510 9520 9530 9540 9550 9560 9570 9580 9590 9600
C A: 1 GTAAACCTGTGCGACTCACAGGTGCTCAAACTGAAATACAGGCTGATTATAGCCTCGCCATCAAGATGTTAACTGCTGTAAGGGATGCCTACTATAACAT
T A: 1 T
T A: 2 T
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 T
T A: 7 G
T A: 8 T
9610 9620 9630 9640 9650 9660 9670 9680 9690 9700
C A: 1 AGGACACAAGTTGAAGGAAGGGGAGACTTATGTTTCAAGAGACCTCCAGTTTATGAGCAAGACCATCCAATCAGAAGGCGTAATGTATCCTGCTGCAATT
T A: 1 T
T A: 2 T
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 T
T A: 7 T
T A: 8 T
9710 9720 9730 9740 9750 9760 9770 9780 9790 9800
C A: 1 AAGAAGGTTCTGCGAGTGGGTCCCTGGATAAACACAATACTGGATGACATCAAGACTAGTATGGAAGCTATTGGAAGTCTCTGTCAAGAGTTAGAATTTC
T A: 1 T
T A: 2 T
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 T
T A: 7 T
T A: 8 T
9810 9820 9830 9840 9850 9860 9870 9880 9890 9900
C A: 1 GAGGGGAGTCATTTGTGACTAGTCTTATAGTTCGGAATTTCTGGTTGTACATCCAGTGCTTTGTCGAGGCAAAAGATCATAGCTTAGCAGGTGCACAAAT
T A: 1 T
T A: 2 T
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 T
T A: 7 T
T A: 8 T
9910 9920 9930 9940 9950 9960 9970 9980 9990 10000
C A: 1 ATCCAATGAGCTTAATAGAACTTTAACCAAAGTTAAGAGATTCTTCAGGTTAGCAGATGAGAGCAGCACCGTTAACTTGTTCATGAACATACCAATGCAG
T A: 1 T
T A: 2 T
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 T
T A: 7 T
T A: 8 T
10010 10020 10030 10040 10050 10060 10070 10080 10090 10100
C A: 1 TTGGGGGGAGGGGACCCTGTTGTTGTTTATAGGAGTTTTTATAGACGGACACCAGATTTTTTGACAGAGGTGTTAACACACATGGAGTTACTACTAACAG
T A: 1 T
T A: 2 G
T A: 3 T
T A: 4 T
T A: 5 T
T A: 6 G
T A: 7 T
T A: 8 T
10110 10120 10130 10140 10150 10160 10170 10180 10190 10200
C A: 1 CAATAGAGATGGATAGAGGAGTTTATATGAACTTCTTCTTAACACTGTTGAACACATGCAAAAATGATAGGGCCACTCTTACCACACTGATGAGAGACCC
T A: 1 A
T A: 2 A
T A: 3 A
T A: 4 A
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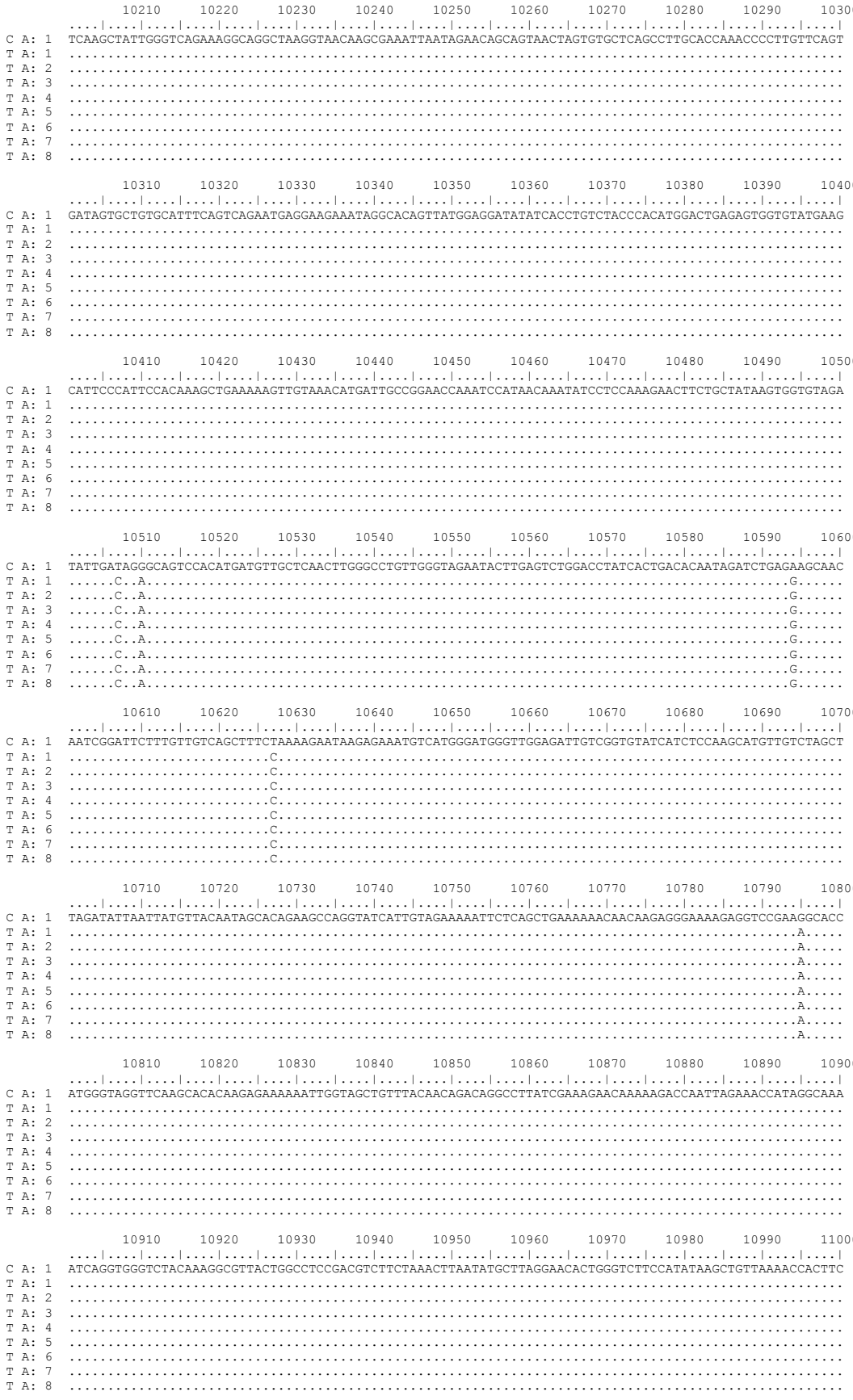
T A: 6 A
T A: 7 A
T A: 8 A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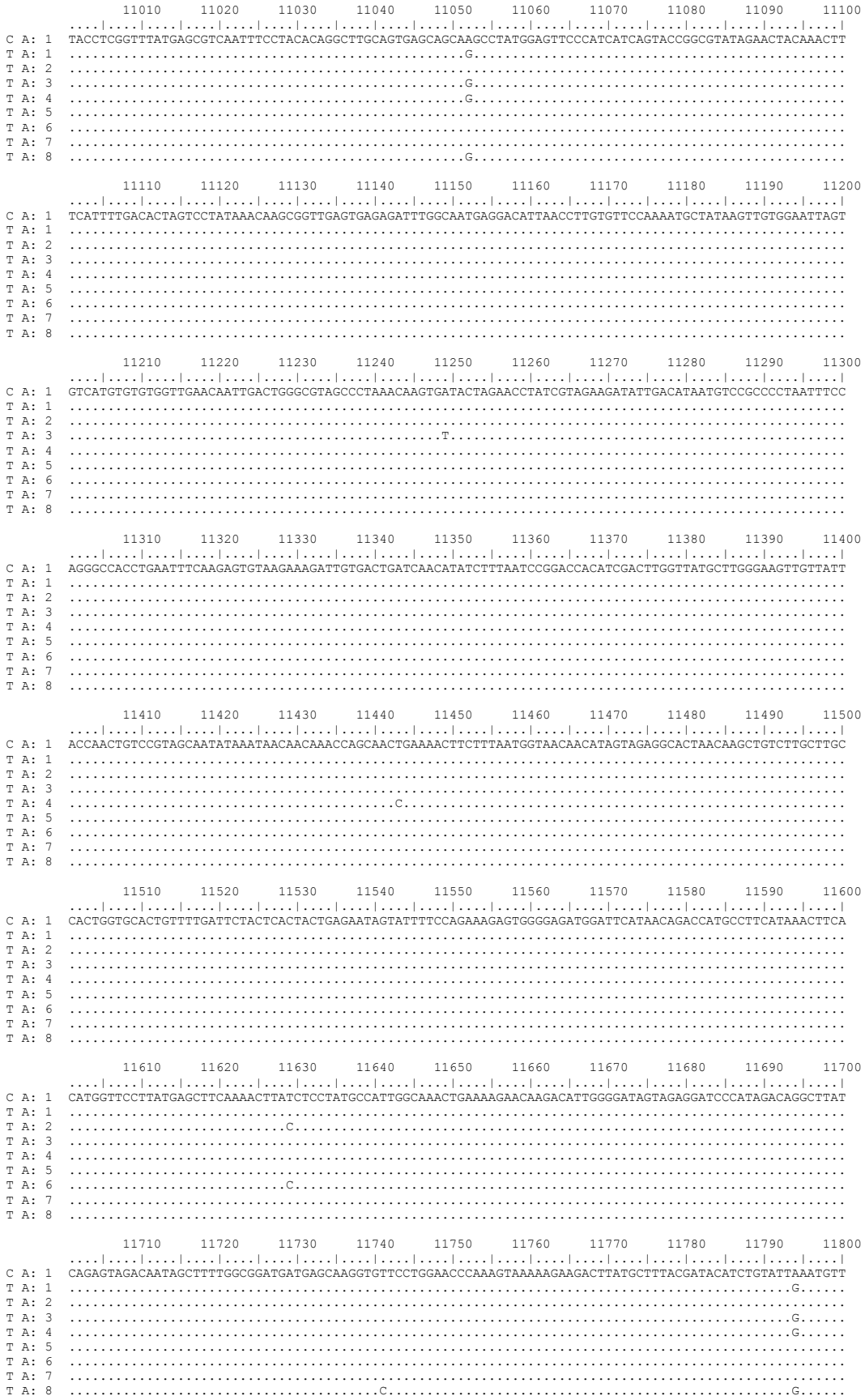
C A: 1 TCAAGCTATTGGGTCAGAAAGGCAGGCTAAGGTAACAAGCGAAATTAATAGAACAGCAGTAACTAGTGTGCTCAGCCTTGCACCAAACCCCTTGTTCAGT
10310 10320 10330 10340 10350 10360 10370 10380 10390 10400
C A: 1 GATAGTGCTGTGCATTTCAGTCAGAATGAGGAAGAAATAGGCACAGTTATGGAGGATATATCACCTGTCTACCCACATGGACTGAGAGTGGTGTATGAAG
10410 10420 10430 10440 10450 10460 10470 10480 10490 10500
C A: 1 CATTCCCATTCCACAAAGCTGAAAAAGTTGTAAACATGATTGCCGGAACCAAATCCATAACAAATATCCTCCAAAGAACTTCTGCTATAAGTGGTGTAGA
10510 10520 10530 10540 10550 10560 10570 10580 10590 10600
C A: 1 TATTGATAGGGCAGTCCACATGATGTTGCTCAACTTGGGCCTGTTGGGTAGAATACTTGAGTCTGGACCTATCACTGACACAATAGATCTGAGAAGCAAC
T A: 1 ......C..A.....G.....
T A: 2 ......C..A.....G.....
T A: 3 ......C..A.....G.....
T A: 4 ......C..A.....G.....
T A: 5 ......C..A.....G.....
T A: 6 ......C..A.....G.....
T A: 7 ......C..A.....G.....
T A: 8 ......C..A.....G.....
10610 10620 10630 10640 10650 10660 10670 10680 10690 10700
C A: 1 AATCGGATTCTTTGTTGTCAGCTTTCTAAAAGAATAAGAGAAATGTCATGGGATGGGTTGGAGATTGTCGGTGTATCATCTCCAAGCATGTTGTCTAGCT
T A: 1 .....C.....
T A: 2 .....C.....
T A: 3 .....C.....
T A: 4 .....C.....
T A: 5 .....C.....
T A: 6 .....C.....
T A: 7 .....C.....
T A: 8 .....C.....
10710 10720 10730 10740 10750 10760 10770 10780 10790 10800
C A: 1 TAGATATTAATTATGTTACAATAGCACAGAAGCCAGGTATCATTGTAGAAAAATTCTCAGCTGAAAAAACAACAAGAGGGAAAAGAGGTCCGAAGGCACC
T A: 1 .....A.....
T A: 2 .....A.....
T A: 3 .....A.....
T A: 4 .....A.....
T A: 5 .....A.....
T A: 6 .....A.....
T A: 7 .....A.....
T A: 8 .....A.....
10810 10820 10830 10840 10850 10860 10870 10880 10890 10900
C A: 1 ATGGGTAGGTTCAAGCACACAAGAGAAAAAATTGGTAGCTGTTTACAACAGACAGGCCTTATCGAAAGAACAAAAAGACCAATTAGAAACCATAGGCAAA
10910 10920 10930 10940 10950 10960 10970 10980 10990 11000
C A: 1 ATCAGGTGGGTCTACAAAGGCGTTACTGGCCTCCGACGTCTTCTAAACTTAATATGCTTAGGAACACTGGGTCTTCCATATAAGCTGTTAAAACCACTTC


11010 11020 11030 11040 11050 11060 11070 11080 11090 11100
C A: 1 TACCTCGGTTTATGAGCGTCAATTTCCTACACAGGCTTGCAGTGAGCAGCAAGCCTATGGAGTTCCCATCATCAGTACCGGCGTATAGAACTACAAACTT
T A: 1 .....G.....
T A: 2 .....
T A: 3 .....G.....
T A: 4 .....G.....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....G.....
11110 11120 11130 11140 11150 11160 11170 11180 11190 11200
C A: 1 TCATTTTGACACTAGTCCTATAAACAAGCGGTTGAGTGAGAGATTTGGCAATGAGGACATTAACCTTGTGTTCCAAAATGCTATAAGTTGTGGAATTAGT
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
11210 11220 11230 11240 11250 11260 11270 11280 11290 11300
C A: 1 GTCATGTGTGTGGTTGAACAATTGACTGGGCGTAGCCCTAAACAAGTGATACTAGAACCTATCGTAGAAGATATTGACATAATGTCCGCCCCTAATTTCC
T A: 1 .....
T A: 2 .....
T A: 3 .....T.....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
11310 11320 11330 11340 11350 11360 11370 11380 11390 11400
C A: 1 AGGGCCACCTGAATTTCAAGAGTGTAAGAAAGATTGTGACTGATCAACATATCTTTAATCCGGACCACATCGACTTGGTTATGCTTGGGAAGTTGTTATT
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
11410 11420 11430 11440 11450 11460 11470 11480 11490 11500
C A: 1 ACCAACTGTCCGTAGCAATATAAATAACAACAAACCAGCAACTGAAAACTTCTTTAATGGTAACAACATAGTAGAGGCACTAACAAGCTGTCTTGCTTGC
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....C.....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
11510 11520 11530 11540 11550 11560 11570 11580 11590 11600
C A: 1 CACTGGTGCACTGTTTTGATTCTACTCACTACTGAGAATAGTATTTTCCAGAAAGAGTGGGGAGATGGATTCATAACAGACCATGCCTTCATAAACTTCA
T A: 1 .....
T A: 2 .....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....
11610 11620 11630 11640 11650 11660 11670 11680 11690 11700
C A: 1 CATGGTTCCTTATGAGCTTCAAAACTTATCTCCTATGCCATTGGCAAACTGAAAAGAACAAGACATTGGGGATAGTAGAGGATCCCATAGACAGGCTTAT
T A: 1 .....
T A: 2 .....C.....
T A: 3 .....
T A: 4 .....
T A: 5 .....
T A: 6 .....C.....
T A: 7 .....
T A: 8 .....
11710 11720 11730 11740 11750 11760 11770 11780 11790 11800
C A: 1 CAGAGTAGACAATAGCTTTTGGCGGATGATGAGCAAGGTGTTCCTGGAACCCAAAGTAAAAAGAAGACTTATGCTTTACGATACATCTGTATTAAATGTT
T A: 1 .....G.....
T A: 2 .....
T A: 3 .....G.....
T A: 4 .....G.....
T A: 5 .....
T A: 6 .....
T A: 7 .....
T A: 8 .....C.....G.....

```
           11810     11820     11830     11840     11850     11860     11870     11880     11890     11900
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CTGGGTAGTATCAGTTTCAAAAACTGGTTTGTTAGCCGGTTAAGAACAGTAGACTACATGGAGATCCCATGGATTGTCAATGCTGAGGGTGACATAGTCG
T A: 1  ..........................................................................................................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................................................
T A: 4  ..........................................................................................................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................................................

           11910     11920     11930     11940     11950     11960     11970     11980     11990     12000
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  AGCAAAAACCTGTCACTGAATATTTGAAAATAATGGCTGCAGGTGTCCATGTAAAAGTCATAATGCTTAGTTACTCTGATATGGCACATGCTATGACACG
T A: 1  ..........................................................................C.......................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................C.......................
T A: 4  ..........................................................................C.......................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................C.......................

           12010     12020     12030     12040     12050     12060     12070     12080     12090     12100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CTTGCTAAGGTGCAAGAACATGCAAGACAATGTGCCCACCATAAAGAAAACAGTATCTCCAACTGATGTCACACCCTCTGTAGACCCCACTCGGGCCCTA
T A: 1  ..........................................................................................................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................................................
T A: 4  ..........................................................................................................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................................................

           12110     12120     12130     12140     12150     12160     12170     12180     12190     12200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CTGCTGTTCCCGAAAGTCATCTTTAGTAAGTTGACCACTTTCAATGCAACATGCAGTACAGCTAAGCAGTCTGGCAGCAATTTGACAAAAAACTACATGA
T A: 1  ..........................................................................................................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................................................
T A: 4  ..........................................................................................................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................................................

           12210     12220     12230     12240     12250     12260     12270     12280     12290     12300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CTTTAATGCCTTGGCACCACATCAACCGTTATAACTTTGTCCATAGCTCCACAGGGTGTAAGGTAAGTGTAAAGGGATGTATAGGCAAGTTGATAACAAA
T A: 1  ..........................................................................................................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................................................
T A: 4  ..........................................................................................................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................................................

           12310     12320     12330     12340     12350     12360     12370     12380     12390     12400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  ACTTGAACTAAGAATTATCTACTTCATCGGTGAGGGAGCTGGGAATCTTATGTCCAGAACTGCATGTGAATATCCTAATCTAAAGTTTGTATACAGGAGT
T A: 1  ..........................................................................C.......................
T A: 2  ..........................................................................C.......................
T A: 3  ..........................................................................C.......................
T A: 4  ..........................................................................C.......................
T A: 5  ..........................................................................C.......................
T A: 6  ..........................................................................C.......................
T A: 7  ..........................................................................C.......................
T A: 8  ..........................................................................C.......................

           12410     12420     12430     12440     12450     12460     12470     12480     12490     12500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CTGAAAGATGCCAATGATCATCATCATCCCACAGAGTATGTGCGTGTAATGGGGAGTATCAGCAGAATAATCGACCTTGGTGAAGGGTTGGCAATGGAAG
T A: 1  ..........................................................................................................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................................................
T A: 4  ..........................................................................................................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................................................

           12510     12520     12530     12540     12550     12560     12570     12580     12590     12600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  CAACTGATGCAACTAAGAGAGAACATTGGGATTTTATCCACAGAGTGTGCAAAGATCCTCTTCTGCTAACCATCTGTGATGCTGAACTAAAAAACATGGA
T A: 1  ..........................................................................................................
T A: 2  ..........................................................................................................
T A: 3  ..........................................................................................................
T A: 4  ..........................................................................................................
T A: 5  ..........................................................................................................
T A: 6  ..........................................................................................................
T A: 7  ..........................................................................................................
T A: 8  ..........................................................................................................
```

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                12610     12620     12630     12640     12650     12660     12670     12680     12690     12700
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  GGACGTCCTGCATTTAGTGATACAGTGGAGGAAACATGTACTATCTTGTAACATATGCACATCATATGGGACTAATGTGTACATGTTTGTTAAGTATCAT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                12710     12720     12730     12740     12750     12760     12770     12780     12790     12800
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  GCACAATCTGAAGCAAAGAAGCTACCACATTTTGTCCGAGTAGTTTCAATATATATCATGCAAGGGAGTAAACTATCAGGATCGGAATGTTATATGTTGT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                12810     12820     12830     12840     12850     12860     12870     12880     12890     12900
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  TAACATTAGGTCACCAGAACATGGCACCTTGCTACGGAGAGGTTAGGTCTGCGAAATCAATCATGGCTTTGACAAGTACTTTTAAACACCCCTGTAGACT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                12910     12920     12930     12940     12950     12960     12970     12980     12990     13000
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  AGACAAACCTGCCATCGAGGCCAACCTGAAATCACTAGCACCTGGGTTGACACTGCCACTTAGTGAGCCTGCTATGCAAAAGTACTTGTTGGATCTTTCA
T A: 1  .......T............................................................................................
T A: 2  .......T............................................................................................
T A: 3  .......T............................................................................................
T A: 4  .......T............................................................................................
T A: 5  .......T............................................................................................
T A: 6  .......T............................................................................................
T A: 7  .......T............................................................................................
T A: 8  .......T............................................................................................

                13010     13020     13030     13040     13050     13060     13070     13080     13090     13100
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  AGCTTAAGTGGAATTAAGAACACGGGGGCAACCATTGGGGGAAGCAAAGTGGTAGAGAAGAAGTGGGCTTTGTGCAAAGTGTCAAATATAGTGAAGTGGT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                13110     13120     13130     13140     13150     13160     13170     13180     13190     13200
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  TAAGTCACGTTCTGAAAATGCCCAAAGGTGATTTAAATTACGATTTTTTCGAGGTGATAGAAAACACATACCCAGACATGGTGAAGCTACTGGACAACTT
T A: 1  ....................................................................................................
T A: 2  ....................................................................................................
T A: 3  ....................................................................................................
T A: 4  ....................................................................................................
T A: 5  ....................................................................................................
T A: 6  ....................................................................................................
T A: 7  ....................................................................................................
T A: 8  ....................................................................................................

                                                                    L stop codon
                13210     13220     13230     13240     13250     13260     13270     13280     13290     13300
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C A: 1  AAACCCGTCGGAGTTGAAAAAGCTCGTCAAAGTCACCGGGTACATCCTGGGTACAAAGTAATAAAGCAATATCAACTCAACACATAATTATCACCTGAAA
T A: 1  ..................................................C.....................................................
T A: 2  ..................................................C.....................................................
T A: 3  ..................................................C.....................................................
T A: 4  ..................................................C.....................................................
T A: 5  ..................................................C.....................................................
T A: 6  ..................................................C.....................................................
T A: 7  ..................................................C.....................................................
T A: 8  ..................................................C.....................................................
```

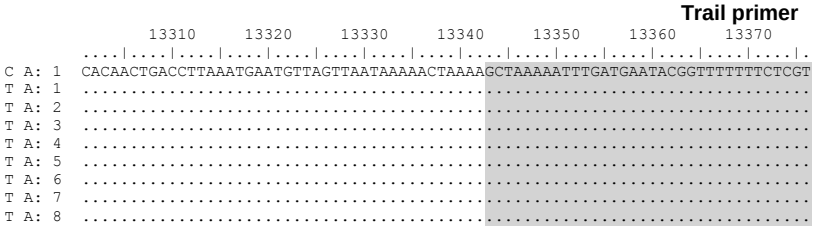


Figure 8. Full nt sequence alignment of subtype A-AMPV isolates. ORF translation start and stop codons are highlighted in green and red respectively. Sequence of the primers at the extreme leader and trailer ends are highlighted in grey as sequence here was not deduced. Ambiguity code Y represents nt C or T.

(ii) Amino acid sequence comparison.

The N, P, M, F, M2:1, M2:2, SH, G and L genes consisted of 391, 278, 254, 538, 186, 73, 174, 391 and 2004 aa residues respectively, in agreement with previous findings (223).

Predicted sequences were similar across all isolates. In the single chicken-derived isolate (CA: 1) 11 amino acids were identified which did not occur in the sequence of the turkey-derived isolates. Three were found in both the P and the F proteins, two in the L protein and one in each of the M, SH and G proteins. Within the turkey-derived isolates, the predicted aa sequence alignments of the N protein were identical, with the exception of the German isolate TA:3, which contained only one aa difference.

In the P protein differences were identified at 3 positions and a single aa in vaccine TA:7 could not be identified due to ambiguities in the nt sequence. Three differences occurred in the M protein, and 12 were seen in the F protein. In the M2:1 and M2:2 proteins, two and four differences were identified respectively. The SH protein alignment revealed individual differences in three positions whilst differences occurred at six positions in the G protein and at eight positions in the L protein.

Between UK field strain TA:1 and its vaccine derivative TA:8 six differences were identified. Of these, four were considered non-conservative mutations occurring in the M protein at aa71, the F protein at aa149 and aa166 and the L protein at

aa1499, whilst two conservative mutations were seen at aa230 in the F protein and at aa131 in the L protein.

Predicted aa differences between the isolates and the positions in which they occur within a protein are shown in table 5. The genome position of the nt responsible for the codon difference is also given as it appears in figure 8. Undetermined amino acids are indicated by an asterisk.

Table 5. aa differences between chicken and turkey-derived AMPV-A isolates. A dash denotes identity with the aa shown for CA:1. An asterisk denotes an undetermined aa due to nt ambiguities.

Gene	aa position	CA: 1	TA: 1	TA: 2	TA: 3	TA: 4	TA: 5	TA: 6	TA: 7	TA: 8	Nt position
N	139	E	-	-	D	-	-	-	-	-	472
P	42	I	-	-	-	-	-	-	*	-	1381-82
	56	S	N	N	N	N	N	N	N	N	1423
	104	K	-	-	E	-	-	-	-	-	1566
	218	I	M	M	M	M	M	M	M	M	1910
	226	A	-	E	-	-	-	-	-	-	1933
	244	R	K	K	K	K	K	K	K	K	1987
	245	V	I	-	I	-	-	-	-	I	1989
M	27	S	-	Y	-	-	-	-	-	-	2198
	71	Q	-	-	-	-	-	-	-	K	2329
	113	K	R	R	R	R	R	R	R	R	2456
	171	D	-	-	N	-	-	-	-	-	2629
F	31	A	V	V	V	V	V	V	V	V	3035
	68	G	D	-	D	-	-	-	-	D	3146
	123	A	-	-	-	T	-	-	-	-	3310
	149	S	-	-	-	-	-	-	-	F	3389
	166	K	-	-	-	-	-	-	-	E	3439
	170	N	S	S	S	-	S	S	S	S	3452
	204	V	-	-	-	-	-	A	-	-	3554
	230	A	-	-	-	-	-	-	-	V	3632
	294	K	-	-	-	-	-	-	E	-	3823
	295	E	-	K	K	-	-	K	-	-	3826
	323	E	-	-	K	-	-	-	-	-	3910
	348	E	-	-	D	-	-	-	-	-	3987
	409	Y	H	H	H	H	H	H	H	H	4168
	435	K	R	R	R	R	R	R	R	R	4247
	453	E	-	-	V	-	-	-	-	-	4301
M2:1	157	K	-	R	-	-	-	R	-	-	5056
	175	S	-	-	-	N	-	-	-	-	5110
M2:2	3	V	-	-	-	M	-	-	-	-	5110
	13	I	-	T	-	-	-	T	-	-	5141
	26	R	K	-	K	-	-	-	-	K	5180
	63	F	-	-	L	-	-	-	-	-	5290
SH	15	W	-	-	-	R	-	-	-	-	5422
	33	A	-	-	-	V	-	-	-	-	5477
	89	T	A	A	A	A	A	A	A	A	5644
	109	C	-	-	-	-	-	-	Y	-	5705
G	133	A	V	V	V	V	V	V	V	V	6381
	248	L	-	-	-	P	-	-	-	-	6726
	250	S	-	-	-	P	-	-	-	-	6731
	271	L	-	-	-	P	-	-	-	-	6795
	275	Y	-	-	-	H	-	-	-	-	6806
	355	N	-	-	-	S	-	-	-	-	7048
	368	N	-	-	Y	-	-	-	-	-	7086
L	5	N	D	D	D	D	D	D	D	D	7259
	131	I	-	-	-	-	-	-	-	L	7637
	294	R	-	-	-	-	-	-	Q	-	8127
	339	V	-	A	-	-	A	A	-	-	8262
	784	N	-	-	-	-	-	-	D	-	9597
	1269	K	R	-	R	R	-	-	-	R	11052
	1335	I	-	-	L	-	-	-	-	-	11249
	1499	F	-	-	-	-	-	-	-	L	11741
	1888	P	S	S	S	S	S	S	S	S	12908
	1953	S	-	-	-	N	-	-	-	-	13104

4.3.4 Comparisons of chicken and turkey-derived subtype B AMPV genomes and deduced amino acid sequences.

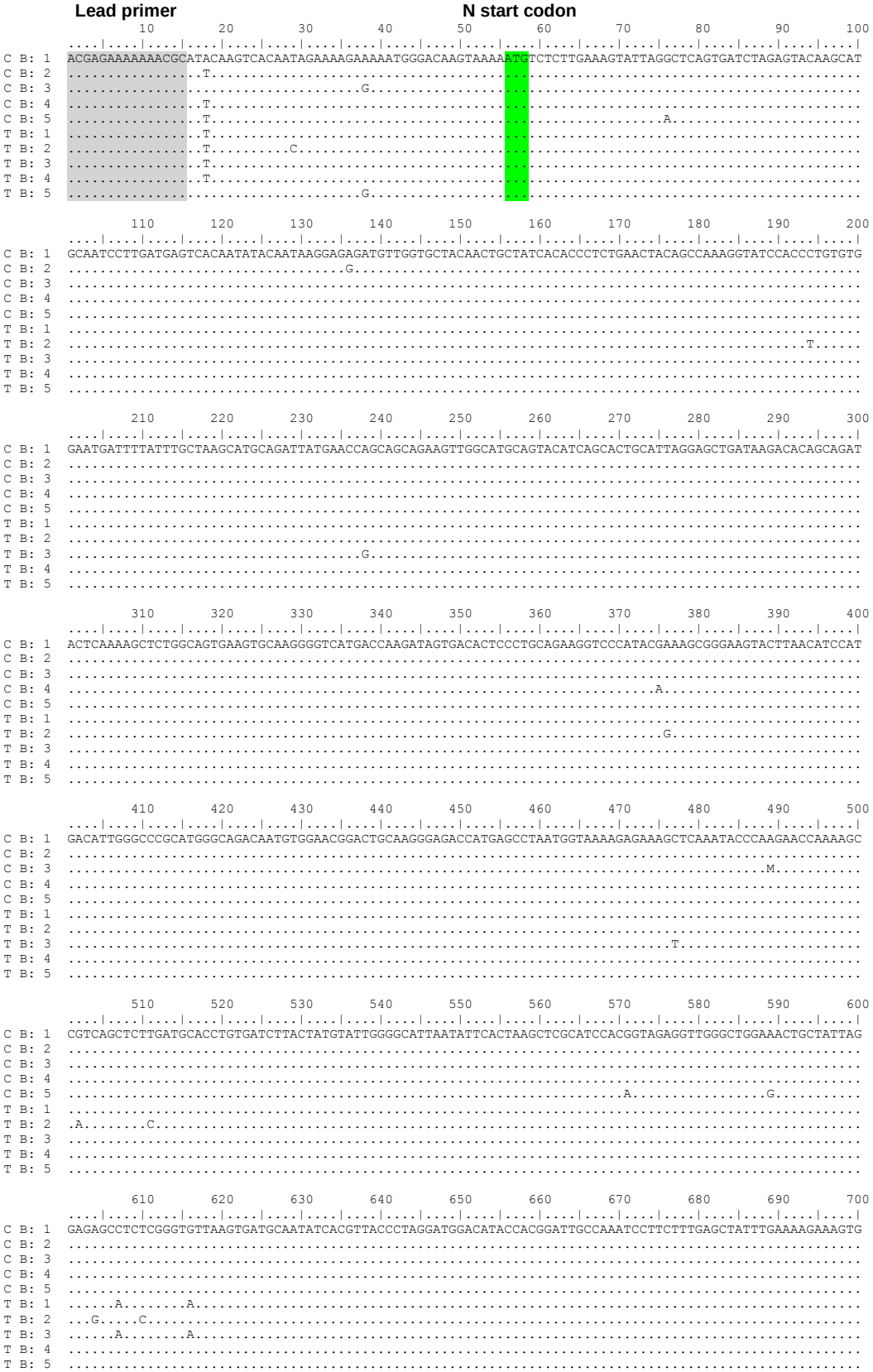
(i) Nucleotide sequence comparison

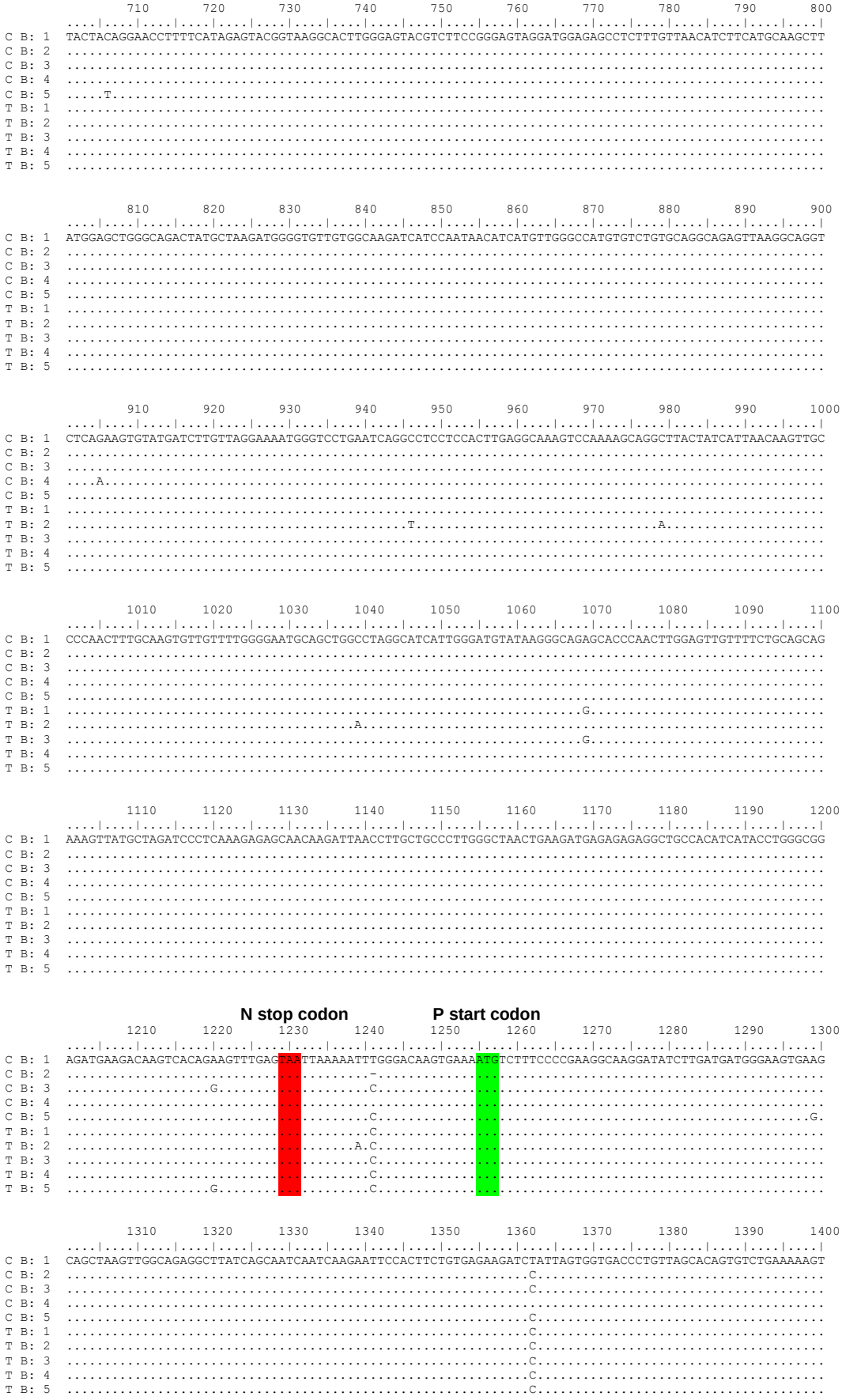
A comparison of chicken and turkey-derived subtype B AMPV isolates identified random individual nt sequence differences, with the highest number occurring in the G ORF. In the non-coding regions nt insertions or deletions were observed, however the non-coding region between P and M was identical. No specific trends could be found that distinguished turkey isolates from chicken isolates.

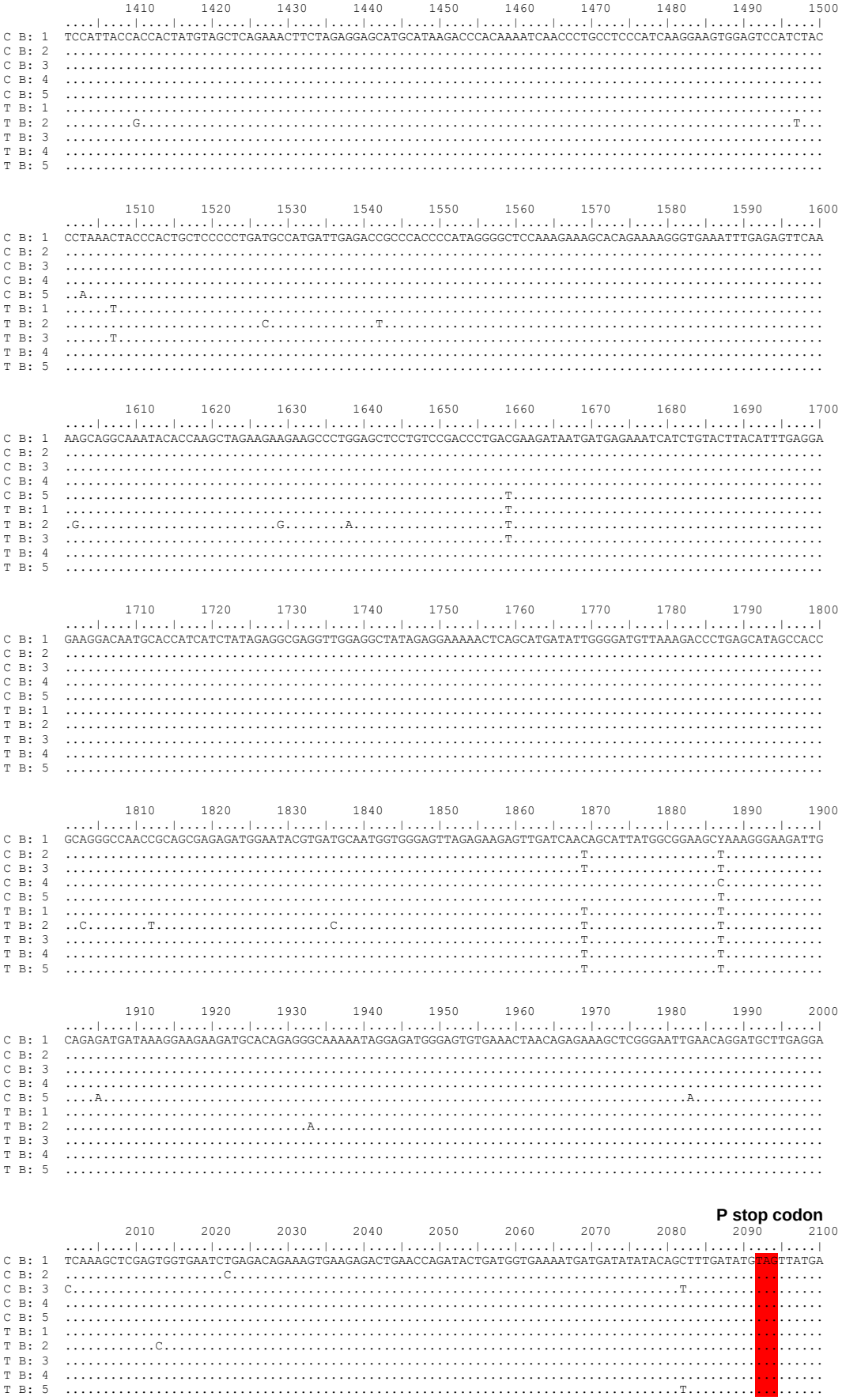
In other findings, the relatively recent Italian field isolate TB:2 contained more individual base differences, deletions and insertions compared with the other turkey isolates. Nucleotide sequences of the Spanish chicken field isolate CB:3 and the turkey-derived vaccine TB:5 were highly similar and shared bases, additions or deletions not observed in other isolates. Nucleotide differences between these two viruses occurred at genome positions 2001 and 6484 although the aa remained the same. Ambiguity codes were used in the sequence of CB:3 at nt positions 489, 6332, 6333, 6334, 6341 and 11434, and in the sequence of TB:5 at nt positions 6499 and 12992, when two nt possibilities were present. Apart from the above mentioned positions, the sequence of the two isolates were identical. The chicken-derived vaccine strain CB:4 possessed two additional nt bases in the M2:2 ORF, compared with all other isolates, which altered the open reading frame and caused an early stop codon. The French chicken-derived field isolate CB:1 was found to be very similar to CB:4, with only nine nt differences, although it did not possess the early stop codon in the M2:2 protein.

The determined nt sequence alignment is shown with the ORF translation start and stop positions highlighted in green and red respectively (figure 9). Undetermined sequence, generated by primers at the extreme leader and trailer ends is highlighted

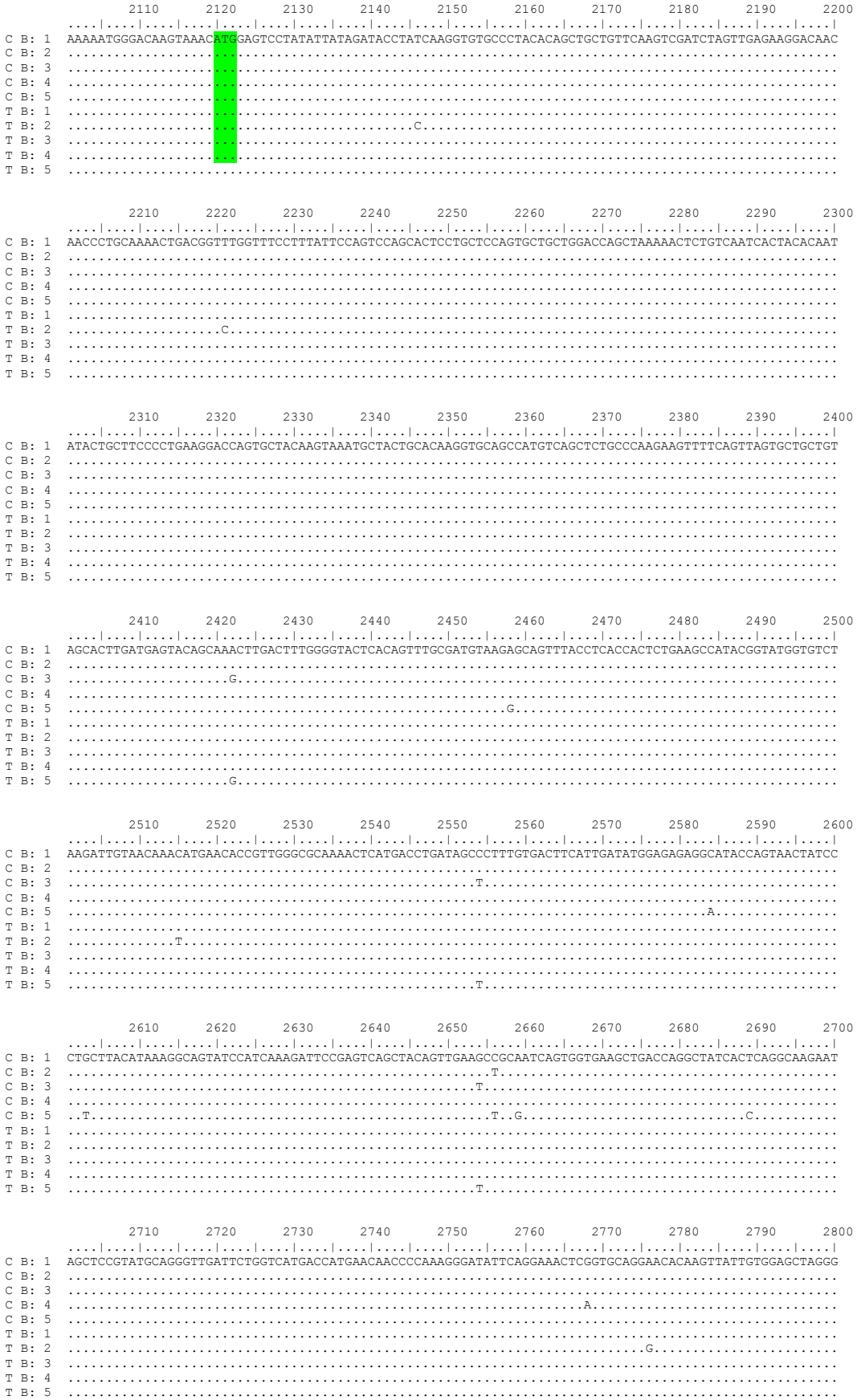
in grey Ambiguity code K represents nt G or T, code M represents nt A or C, code R represents nt A or G and code Y represents nt C or T.

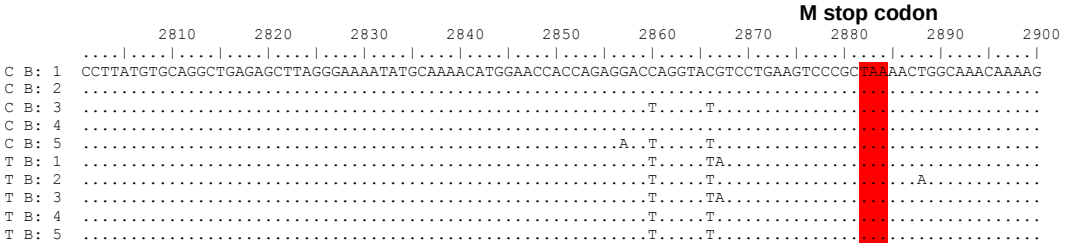


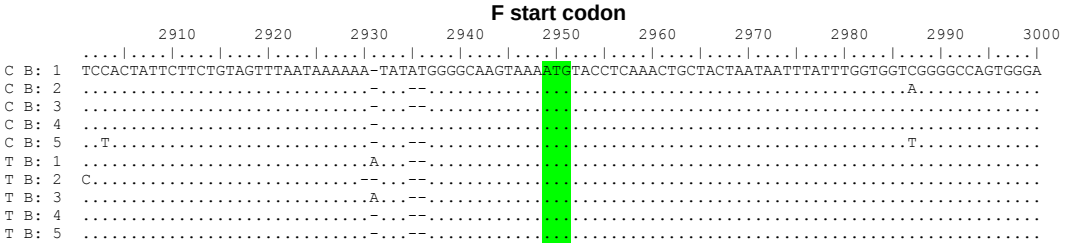

N stop codon
P start codon

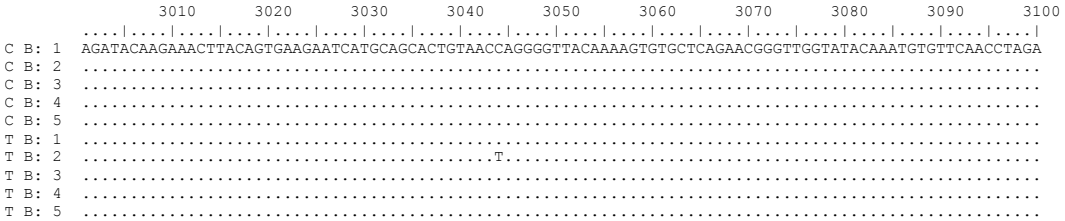

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
C B: 1 TCCATTACCACCACTATGTAGCTCAGAAACTTCTAGAGGAGCATGCATAAGACCCACAAAATCAACCCTGCCTCCCATCAAGGAAGTGGAGTCCATCTAC
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....G.....T...
T B: 3 .....
T B: 4 .....
T B: 5 .....
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
C B: 1 CCTAAACTACCCACTGCTCCCCCTGATGCCATGATTGAGACCGCCCACCCCATAGGGGCTCCAAAGAAAGCACAGAAAAGGGTGAAATTTGAGAGTTCAA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 ..A.....
T B: 1 ......T.....
T B: 2 .....C.....T.....
T B: 3 ......T.....
T B: 4 .....
T B: 5 .....
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
C B: 1 AAGCAGGCAAATACACCAAGCTAGAAGAAGAAGCCCTGGAGCTCCTGTCCGACCCTGACGAAGATAATGATGAGAAATCATCTGTACTTACATTTGAGGA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....T.....
T B: 1 .....T.....
T B: 2 .G.....G.....A.....T.....
T B: 3 .....T.....
T B: 4 .....
T B: 5 .....
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
C B: 1 GAAGGACAATGCACCATCATCTATAGAGGCGAGGTTGGAGGCTATAGAGGAAAAACTCAGCATGATATTGGGGATGTTAAAGACCCTGAGCATAGCCACC
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
C B: 1 GCAGGGCCAACCGCAGCGAGAGATGGAATACGTGATGCAATGGTGGGAGTTAGAGAAGAGTTGATCAACAGCATTATGGCGGAAGCYAAAGGGAAGATTG
C B: 2 .....T.....T.....
C B: 3 .....T.....T.....
C B: 4 .....C.....
C B: 5 .....T.....
T B: 1 .....T.....T.....
T B: 2 ..C.....T.....C.....T.....T.....
T B: 3 .....T.....T.....
T B: 4 .....T.....T.....
T B: 5 .....T.....T.....
1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
C B: 1 CAGAGATGATAAAGGAAGAAGATGCACAGAGGGCAAAAATAGGAGATGGGAGTGTGAAACTAACAGAGAAAGCTCGGGAATTGAACAGGATGCTTGAGGA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 ....A.....A.....
T B: 1 .....
T B: 2 .....A.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
P stop codon
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
C B: 1 TCAAAGCTCGAGTGGTGAATCTGAGACAGAAAGTGAAGAGACTGAACCAGATACTGATGGTGAAAATGATGATATATACAGCTTTGATATGTAGTTATGA
C B: 2 .....C.....
C B: 3 C.....T.....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....C.....
T B: 3 .....
T B: 4 .....
T B: 5 .....T.....

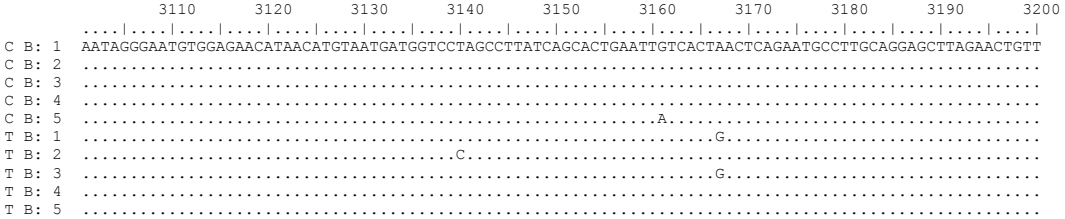
M start codon

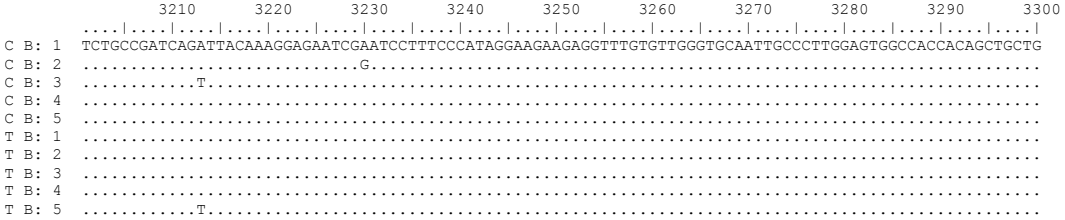


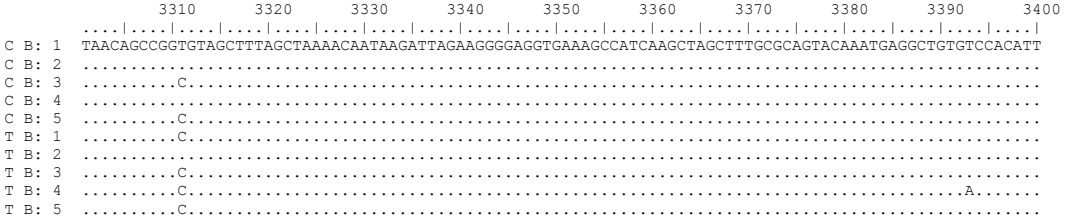

M stop codon
2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
C B: 1 CCTTATGTGCAGGCTGAGAGCTTAGGGAAAATATGCAAAACATGGAACCACCAGAGGACCAGGTACGTCCTGAAGTCCCGCTAAAACTGGCAAACAAAAG
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

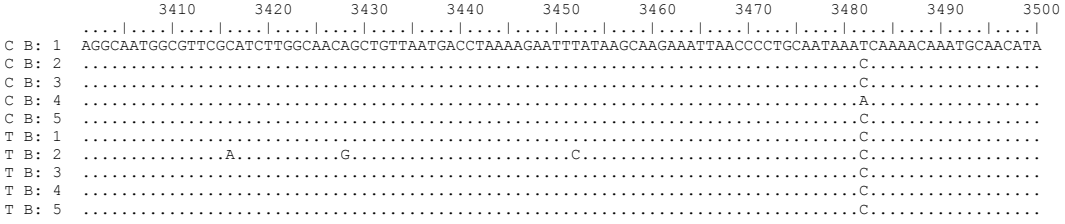

F start codon
2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
C B: 1 TCCACTATTCTTCTGTAGTTTAATAAAAAA-TATATGGGGCAAGTAAAATGTACCTCAAACTGCTACTAATAATTTATTTGGTGGTCGGGGCCAGTGGGA
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

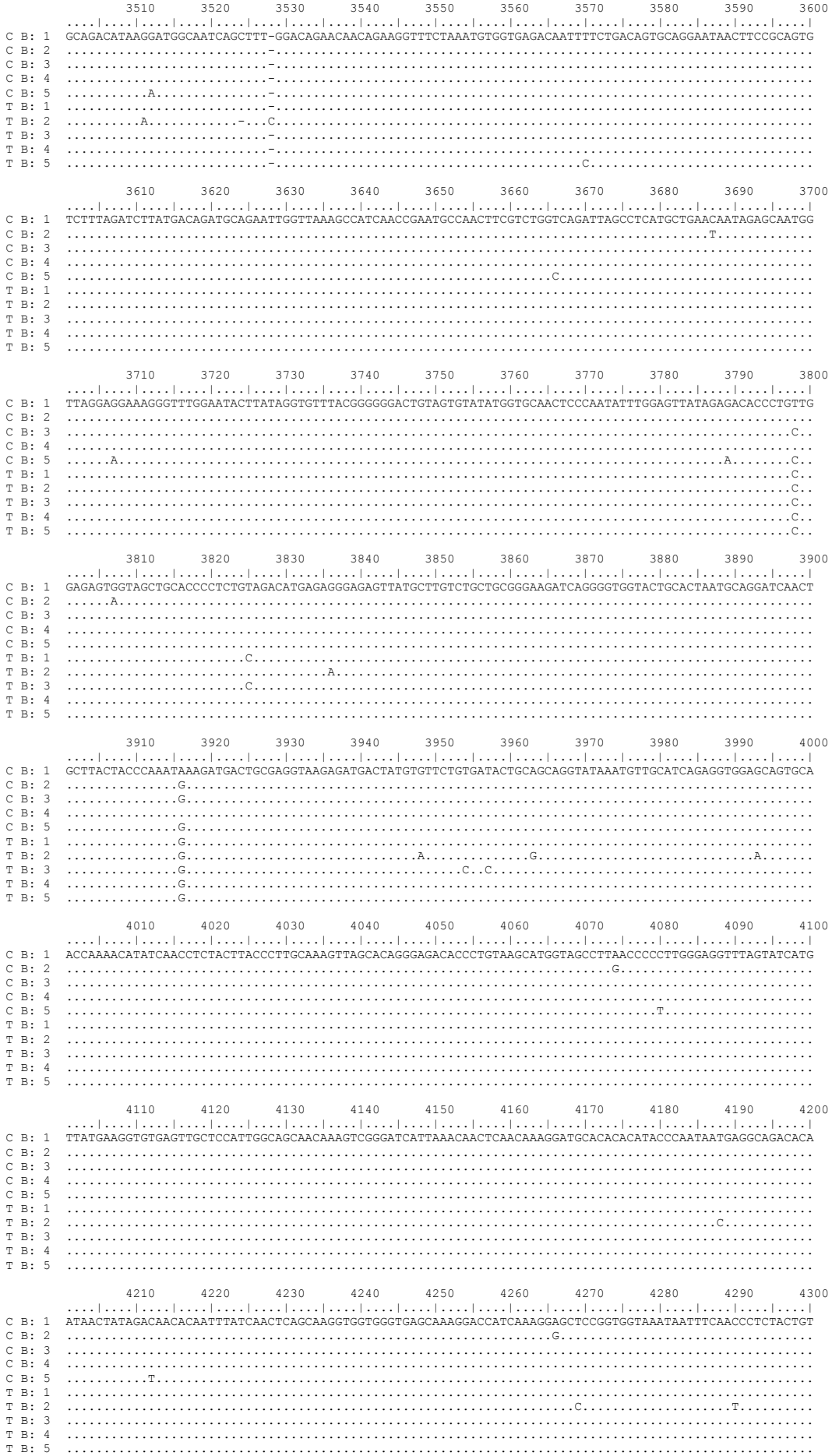

3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
C B: 1 AGATACAAGAAACTTACAGTGAAGAATCATGCAGCACTGTAACCAGGGGTTACAAAAGTGTGCTCAGAACGGGTTGGTATACAAATGTGTTCAACCTAGA
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

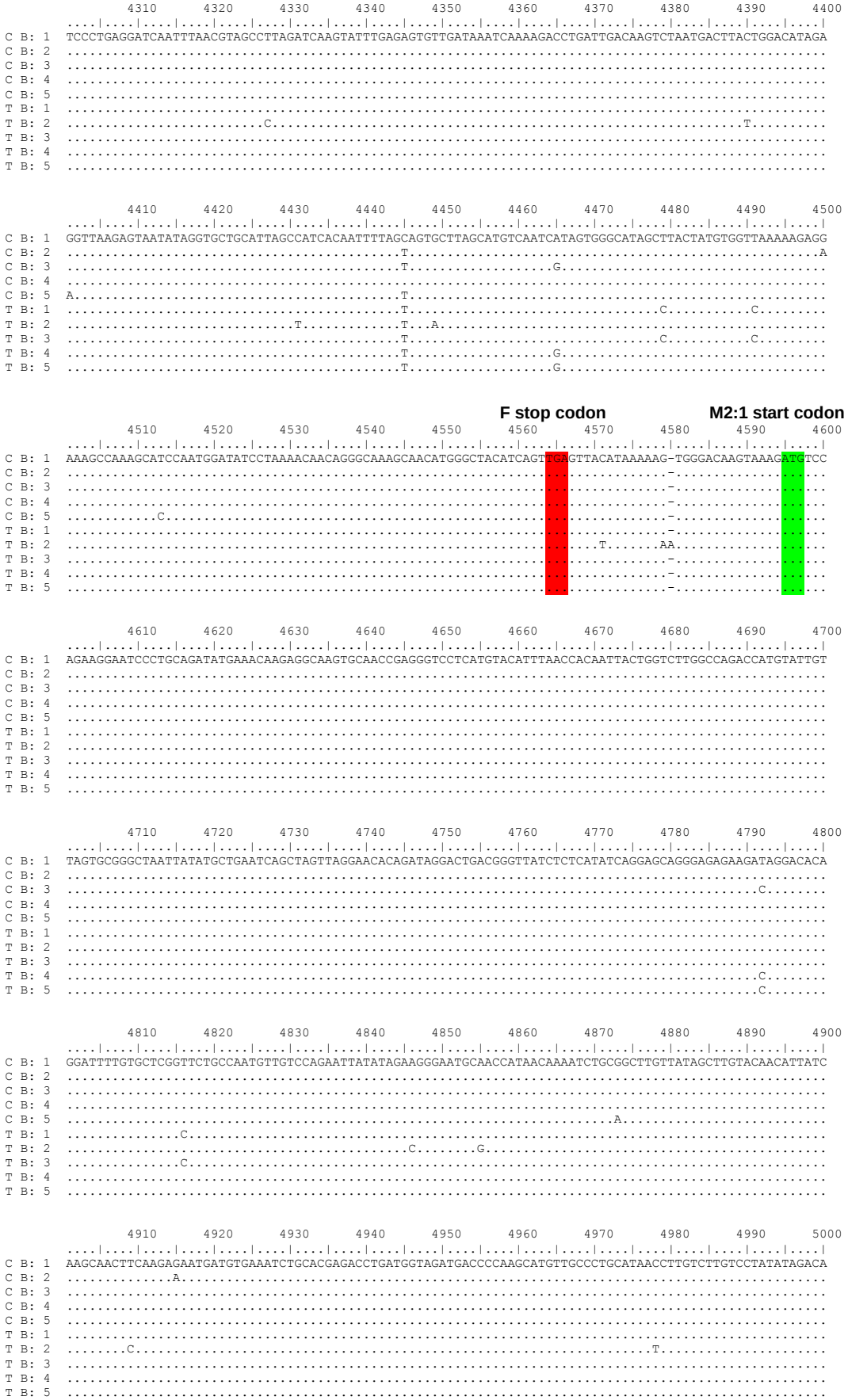

3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
C B: 1 AATAGGGAATGTGGAGAACATAACATGTAATGATGGTCCTAGCCTTATCAGCACTGAATTGTCACTAACTCAGAATGCCTTGCAGGAGCTTAGAACTGTT
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

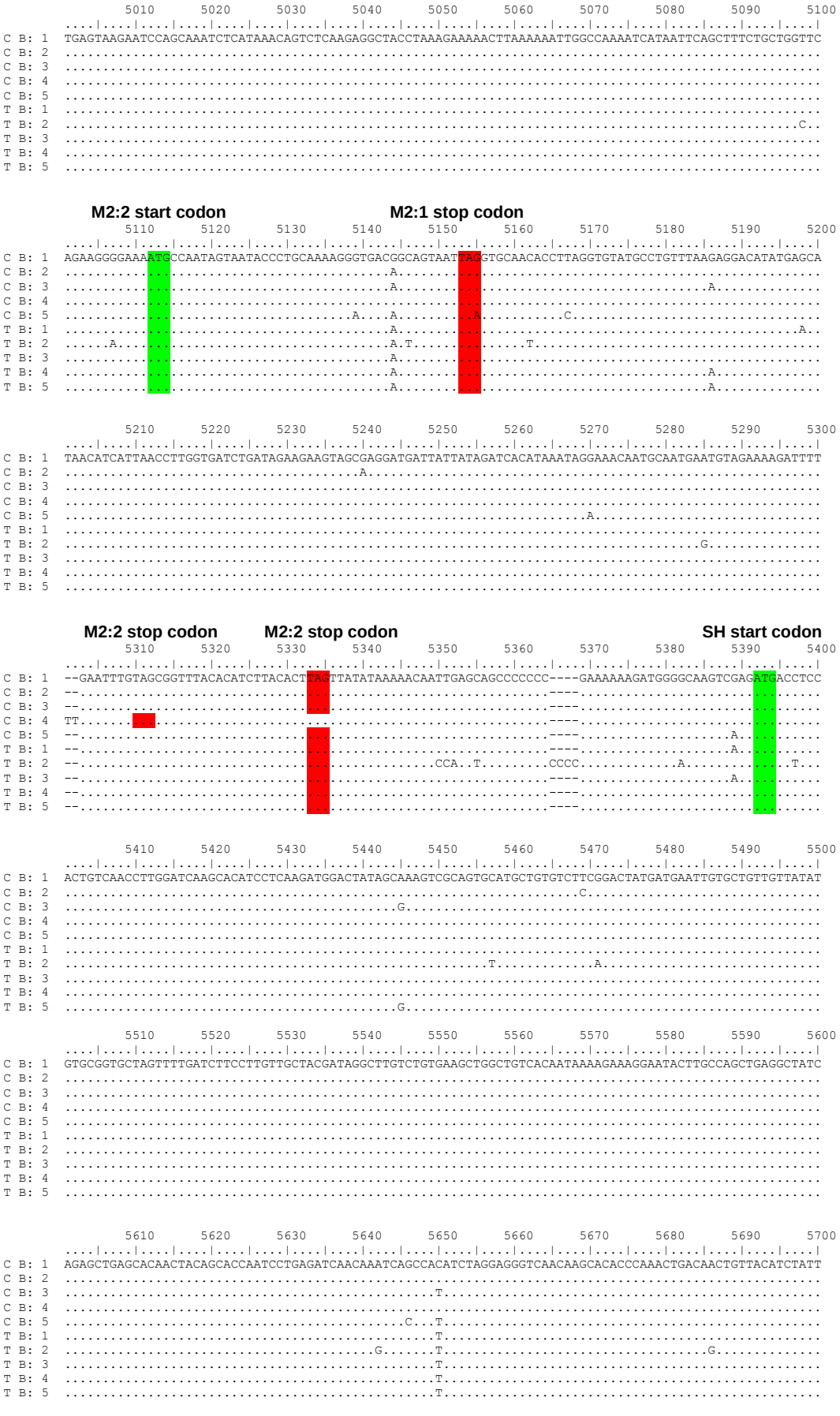

3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
C B: 1 TCTGCCGATCAGATTACAAAGGAGAATCGAATCCTTTCCCATAGGAAGAAGAGGTTTGTGTTGGGTGCAATTGCCCTTGGAGTGGCCACCACAGCTGCTG
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5


3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
C B: 1 TAACAGCCGGTGTAGCTTTAGCTAAAACAATAAGATTAGAAGGGGAGGTGAAAGCCATCAAGCTAGCTTTGCGCAGTACAAATGAGGCTGTGTCCACATT
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

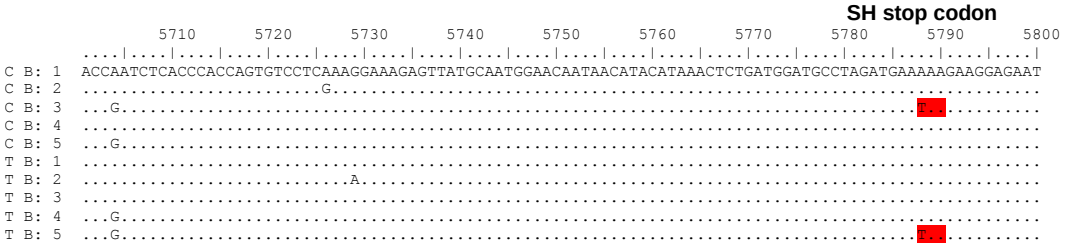

3410 3420 3430 3440 3450 3460 3470 3480 3490 3500
C B: 1 AGGCAATGGCGTTCGCATCTTGGCAACAGCTGTTAATGACCTAAAAGAATTTATAAGCAAGAAATTAACCCCTGCAATAAATCAAAACAAATGCAACATA
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

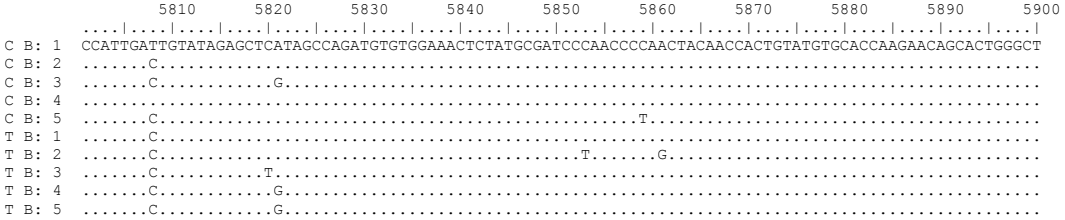

3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
C B: 1 GCAGACATAAGGATGGCAATCAGCTTT-GGACAGAACAACAGAAGGTTTCTAAATGTGGTGAGACAATTTTCTGACAGTGCAGGAATAACTTCCGCAGTG
C B: 2 .....-.....
C B: 3 .....-.....
C B: 4 .....-.....
C B: 5 ..........A.....-.....
T B: 1 .....-.....
T B: 2 ..........A.....-...C.....
T B: 3 .....-.....
T B: 4 .....-.....
T B: 5 .....-.....C.....
3610 3620 3630 3640 3650 3660 3670 3680 3690 3700
C B: 1 TCTTTAGATCTTATGACAGATGCAGAATTGGTTAAAGCCATCAACCGAATGCCAACTTCGTCTGGTCAGATTAGCCTCATGCTGAACAATAGAGCAATGG
C B: 2 .....T.....
C B: 3 .....
C B: 4 .....
C B: 5 .....C.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
3710 3720 3730 3740 3750 3760 3770 3780 3790 3800
C B: 1 TTAGGAGGAAAGGGTTTGGAATACTTATAGGTGTTTACGGGGGGACTGTAGTGTATATGGTGCAACTCCCAATATTTGGAGTTATAGAGACACCCTGTTG
C B: 2 .....
C B: 3 .....C..
C B: 4 .....
C B: 5 ......A.....A.....C..
T B: 1 .....C..
T B: 2 .....C..
T B: 3 .....C..
T B: 4 .....C..
T B: 5 .....C..
3810 3820 3830 3840 3850 3860 3870 3880 3890 3900
C B: 1 GAGAGTGGTAGCTGCACCCCTCTGTAGACATGAGAGGGAGAGTTATGCTTGTCTGCTGCGGGAAGATCAGGGGTGGTACTGCACTAATGCAGGATCAACT
C B: 2 ......A.....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....C.....
T B: 2 .....A.....
T B: 3 .....C.....
T B: 4 .....
T B: 5 .....
3910 3920 3930 3940 3950 3960 3970 3980 3990 4000
C B: 1 GCTTACTACCCAAATAAAGATGACTGCGAGGTAAGAGATGACTATGTGTTCTGTGATACTGCAGCAGGTATAAATGTTGCATCAGAGGTGGAGCAGTGCA
C B: 2 .....G.....
C B: 3 .....G.....
C B: 4 .....
C B: 5 .....G.....
T B: 1 .....G.....
T B: 2 .....G.....A.....G.....A.....
T B: 3 .....G.....C..C.....
T B: 4 .....G.....
T B: 5 .....G.....
4010 4020 4030 4040 4050 4060 4070 4080 4090 4100
C B: 1 ACCAAAACATATCAACCTCTACTTACCCTTGCAAAGTTAGCACAGGGAGACACCCTGTAAGCATGGTAGCCTTAACCCCCTTGGGAGGTTTAGTATCATG
C B: 2 .....G.....
C B: 3 .....
C B: 4 .....
C B: 5 .....T.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
4110 4120 4130 4140 4150 4160 4170 4180 4190 4200
C B: 1 TTATGAAGGTGTGAGTTGCTCCATTGGCAGCAACAAAGTCGGGATCATTAAACAACTCAACAAAGGATGCACACACATACCCAATAATGAGGCAGACACA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....C.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
4210 4220 4230 4240 4250 4260 4270 4280 4290 4300
C B: 1 ATAACTATAGACAACACAATTTATCAACTCAGCAAGGTGGTGGGTGAGCAAAGGACCATCAAAGGAGCTCCGGTGGTAAATAATTTCAACCCTCTACTGT
C B: 2 .....G.....
C B: 3 .....
C B: 4 .....
C B: 5 ....T.....
T B: 1 .....
T B: 2 .....C.....T.....
T B: 3 .....
T B: 4 .....
T B: 5 .....


4310 4320 4330 4340 4350 4360 4370 4380 4390 4400
C B: 1 TCCCTGAGGATCAATTTAACGTAGCCTTAGATCAAGTATTTGAGAGTGTTGATAAATCAAAAGACCTGATTGACAAGTCTAATGACTTACTGGACATAGA
4410 4420 4430 4440 4450 4460 4470 4480 4490 4500
C B: 1 GGTTAAGAGTAATATAGGTGCTGCATTAGCCATCACAATTTTAGCAGTGCTTAGCATGTCAATCATAGTGGGCATAGCTTACTATGTGGTTAAAAAGAGG
F stop codon
M2:1 start codon
4510 4520 4530 4540 4550 4560 4570 4580 4590 4600
C B: 1 AAAGCCAAAGCATCCAATGGATATCCTAAAACAACAGGGCAAAGCAACATGGGCTACATCAGTTGAGTTACATAAAAAG-TGGGACAAGTAAAGATGTCC
4610 4620 4630 4640 4650 4660 4670 4680 4690 4700
C B: 1 AGAAGGAATCCCTGCAGATATGAAACAAGAGGCAAGTGCAACCGAGGGTCCTCATGTACATTTAACCACAATTACTGGTCTTGGCCAGACCATGTATTGT
4710 4720 4730 4740 4750 4760 4770 4780 4790 4800
C B: 1 TAGTGCGGGCTAATTATATGCTGAATCAGCTAGTTAGGAACACAGATAGGACTGACGGGTTATCTCTCATATCAGGAGCAGGGAGAGAAGATAGGACACA
4810 4820 4830 4840 4850 4860 4870 4880 4890 4900
C B: 1 GGATTTTGTGCTCGGTTCTGCCAATGTTGTCCAGAATTATATAGAAGGGAATGCAACCATAACAAAATCTGCGGCTTGTTATAGCTTGTACAACATTATC
4910 4920 4930 4940 4950 4960 4970 4980 4990 5000
C B: 1 AAGCAACTTCAAGAGAATGATGTGAAATCTGCACGAGACCTGATGGTAGATGACCCCAAGCATGTTGCCCTGCATAACCTTGTCTTGTCCTATATAGACA

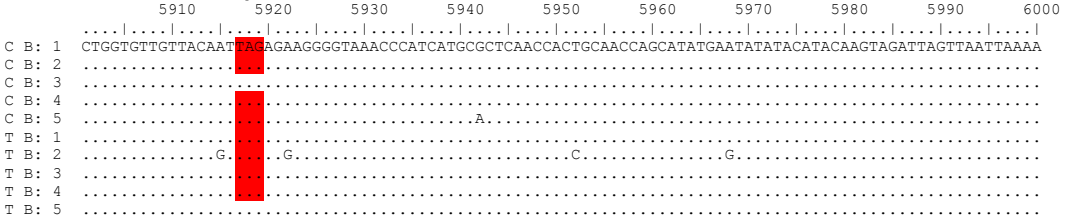

M2:2 start codon
M2:1 stop codon
M2:2 stop codon
M2:2 stop codon
SH start codon

SH stop codon

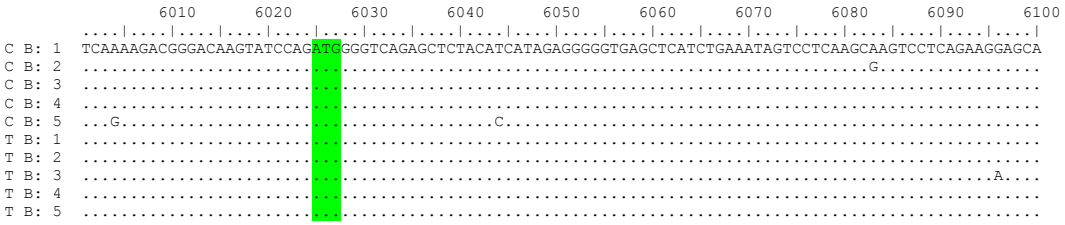


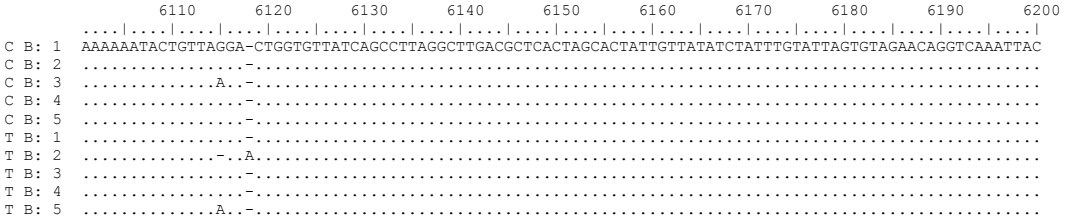


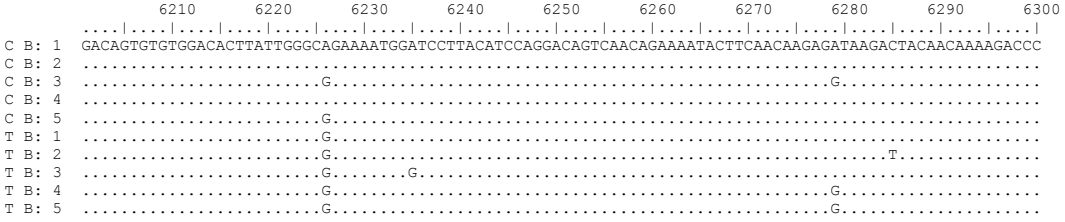
SH stop codon

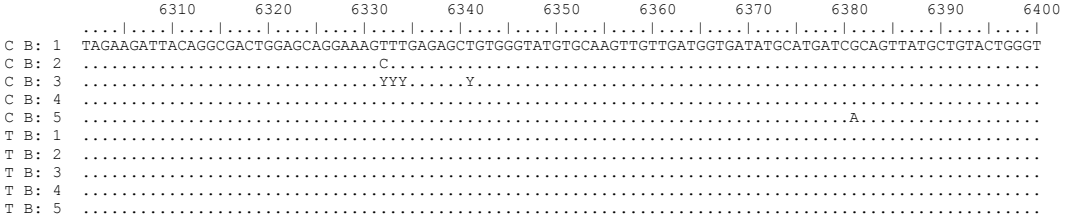


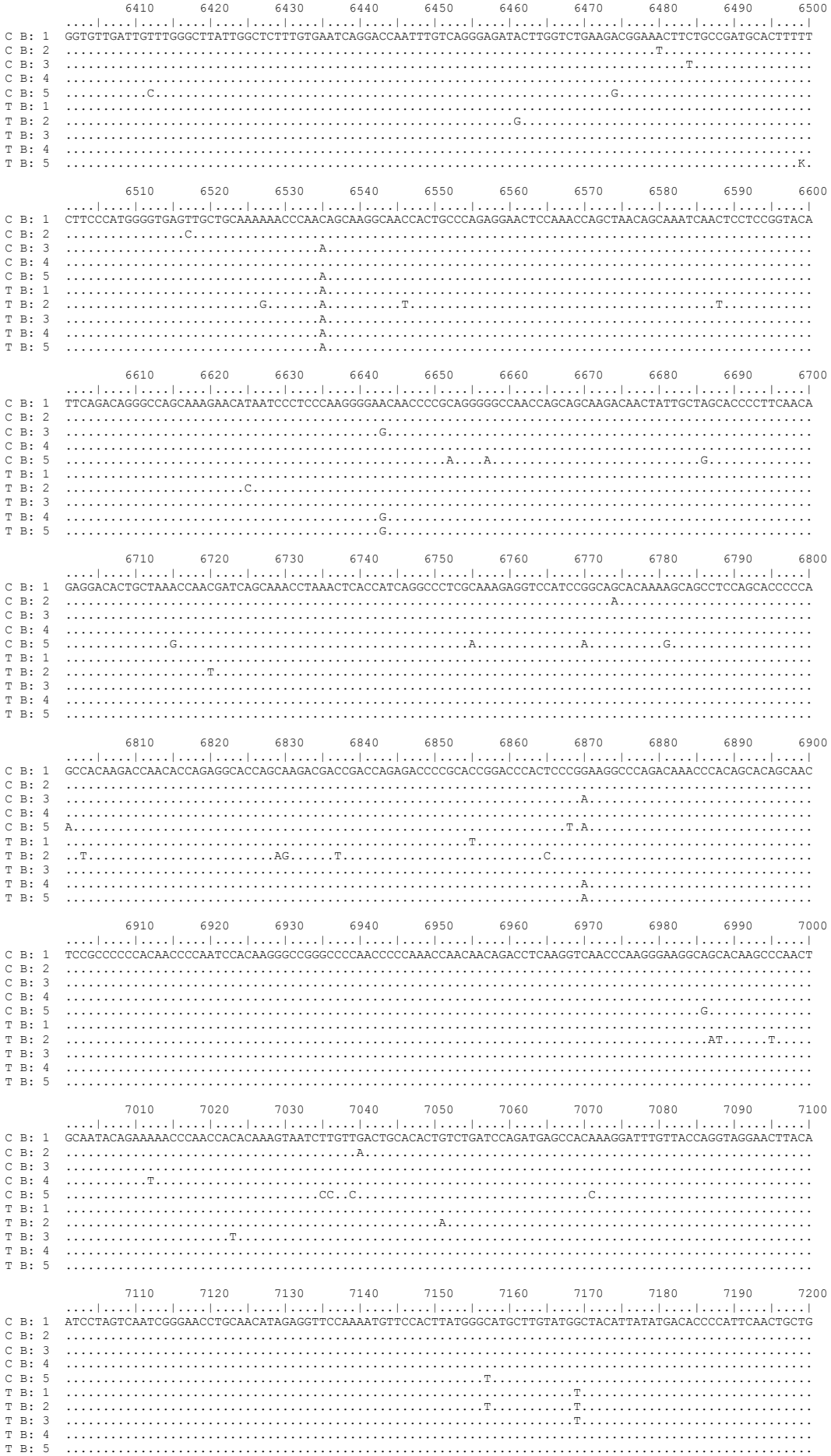
G start codon

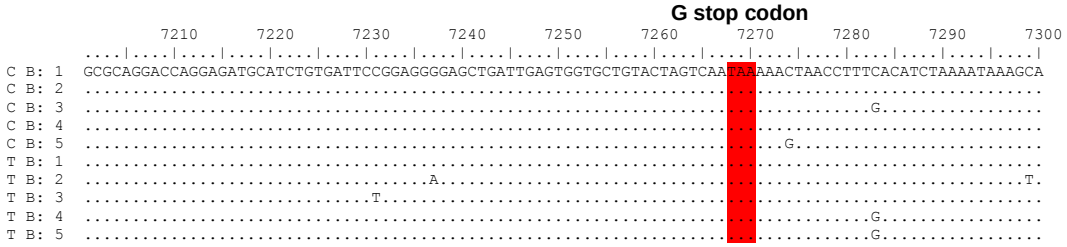


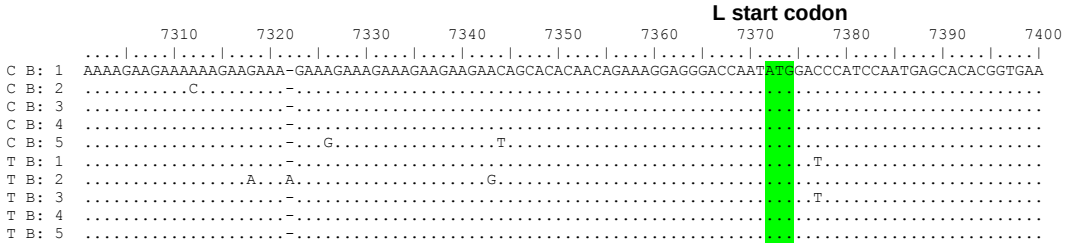


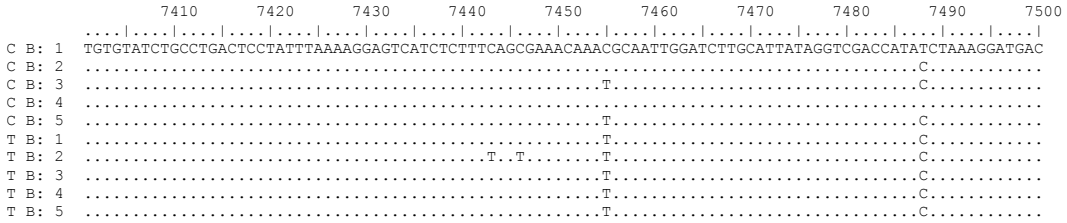


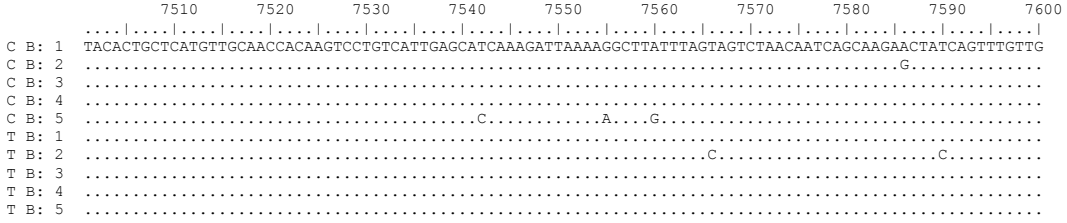


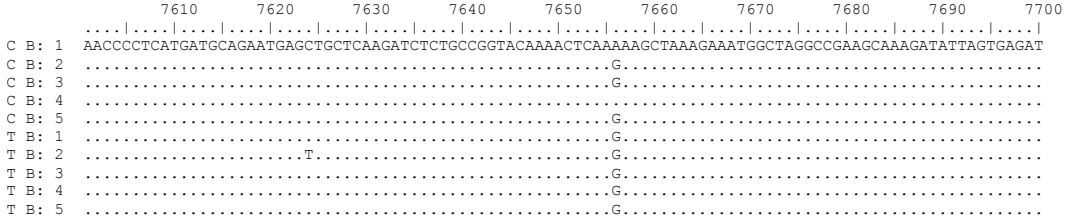

6410 6420 6430 6440 6450 6460 6470 6480 6490 6500
C B: 1 GGTGTTGATTGTTTGGGCTTATTGGCTCTTTGTGAATCAGGACCAATTTGTCAGGGAGATACTTGGTCTGAAGACGGAAACTTCTGCCGATGCACTTTTT
C B: 2 ....T.....
C B: 3 ....T.....
C B: 4 ....
C B: 5 ..........C.....G.....
T B: 1 ....
T B: 2 ....G.....
T B: 3 ....
T B: 4 ....
T B: 5 ....K.
6510 6520 6530 6540 6550 6560 6570 6580 6590 6600
C B: 1 CTTCCCATGGGGTGAGTTGCTGCAAAAAACCCAACAGCAAGGCAACCACTGCCCAGAGGAACTCCAAACCAGCTAACAGCAAATCAACTCCTCCGGTACA
C B: 2 ....C.....
C B: 3 ....A.....
C B: 4 ....
C B: 5 ....A.....
T B: 1 ....A.....
T B: 2 ..........G.....A.....T.....T.....
T B: 3 ....A.....
T B: 4 ....A.....
T B: 5 ....A.....
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700
C B: 1 TTCAGACAGGGCCAGCAAAGAACATAATCCCTCCCAAGGGGAACAACCCCGCAGGGGGCCAACCAGCAGCAAGACAACTATTGCTAGCACCCCTTCAACA
C B: 2 ....
C B: 3 ....G.....
C B: 4 ....
C B: 5 ..........A...A.....G.....
T B: 1 ....
T B: 2 ....C.....
T B: 3 ....
T B: 4 ....G.....
T B: 5 ....G.....
6710 6720 6730 6740 6750 6760 6770 6780 6790 6800
C B: 1 GAGGACACTGCTAAACCAACGATCAGCAAACCTAAACTCACCATCAGGCCCTCGCAAAGAGGTCCATCCGGCAGCACAAAAGCAGCCTCCAGCACCCCCA
C B: 2 ....A.....
C B: 3 ....
C B: 4 ....
C B: 5 ..........G.....A.....A.....G.....
T B: 1 ....
T B: 2 ....T.....
T B: 3 ....
T B: 4 ....
T B: 5 ....
6810 6820 6830 6840 6850 6860 6870 6880 6890 6900
C B: 1 GCCACAAGACCAACACCAGAGGCACCAGCAAGACGACCGACCAGAGACCCCGCACCGGACCCACTCCCGGAAGGCCCAGACAAACCCACAGCACAGCAAC
C B: 2 ....
C B: 3 ....A.....
C B: 4 ....
C B: 5 A.....T.A.....
T B: 1 ....T.....
T B: 2 ..T.....AG.....T.....C.....
T B: 3 ....
T B: 4 ....A.....
T B: 5 ....A.....
6910 6920 6930 6940 6950 6960 6970 6980 6990 7000
C B: 1 TCCGCCCCCCACAACCCCAATCCACAAGGGCCGGGCCCCAACCCCCAAACCAACAACAGACCTCAAGGTCAACCCAAGGGAAGGCAGCACAAGCCCAACT
C B: 2 ....
C B: 3 ....
C B: 4 ....
C B: 5 ....G.....
T B: 1 ....
T B: 2 ..........AT.....T.....
T B: 3 ....
T B: 4 ....
T B: 5 ....
7010 7020 7030 7040 7050 7060 7070 7080 7090 7100
C B: 1 GCAATACAGAAAAACCCAACCACACAAAGTAATCTTGTTGACTGCACACTGTCTGATCCAGATGAGCCACAAAGGATTTGTTACCAGGTAGGAACTTACA
C B: 2 ....A.....
C B: 3 ....
C B: 4 ....T.....
C B: 5 ..........CC..C.....C.....
T B: 1 ....
T B: 2 ....A.....
T B: 3 ....T.....
T B: 4 ....
T B: 5 ....
7110 7120 7130 7140 7150 7160 7170 7180 7190 7200
C B: 1 ATCCTAGTCAATCGGGAACCTGCAACATAGAGGTTCCAAAATGTTCCACTTATGGGCATGCTTGTATGGCTACATTATATGACACCCCATTCAACTGCTG
C B: 2 ....
C B: 3 ....
C B: 4 ....
C B: 5 ....T.....
T B: 1 ....T.....
T B: 2 ..........T.....T.....
T B: 3 ....T.....
T B: 4 ....
T B: 5 ....

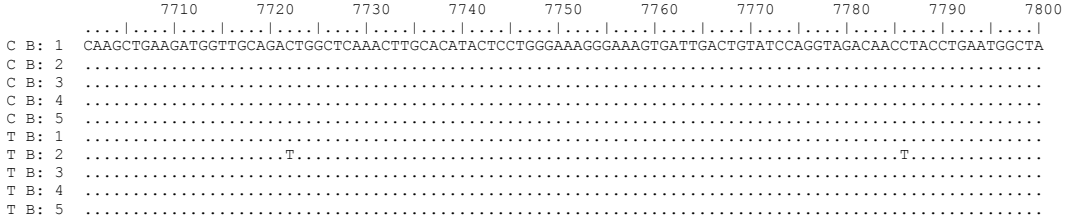

G stop codon
7210 7220 7230 7240 7250 7260 7270 7280 7290 7300
C B: 1 GCGCAGGACCAGGAGATGCATCTGTGATTCCGGAGGGGAGCTGATTGAGTGGTGCTGTACTAGTCAATAAAAACTAACCTTTCACATCTAAAATAAAGCA
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

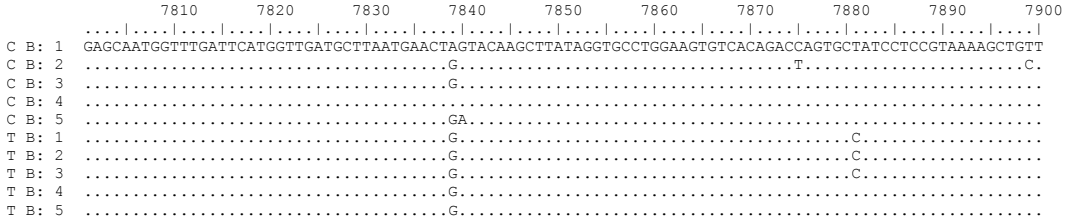

L start codon
7310 7320 7330 7340 7350 7360 7370 7380 7390 7400
C B: 1 AAAAGAAGAAAAAAGAAGAAA-GAAAGAAAGAAAGAAGAAGAACAGCACACAACAGAAAGGAGGGACCAATATGGACCCATCCAATGAGCACACGGTGAA
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

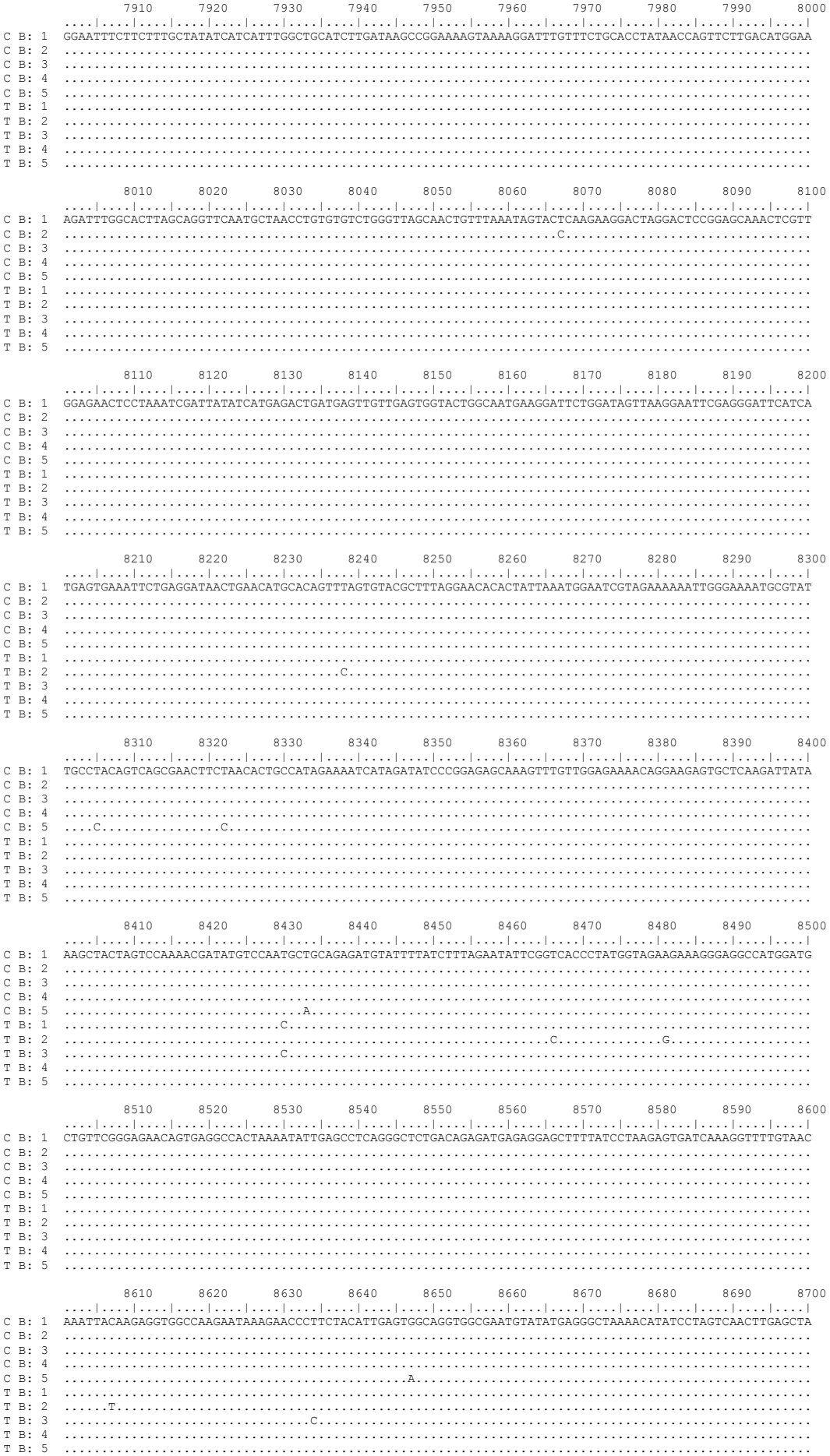

7410 7420 7430 7440 7450 7460 7470 7480 7490 7500
C B: 1 TGTGTATCTGCCTGACTCCTATTTAAAAGGAGTCATCTCTTTCAGCGAAACAAACGCAATTGGATCTTGCATTATAGGTCGACCATATCTAAAGGATGAC
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

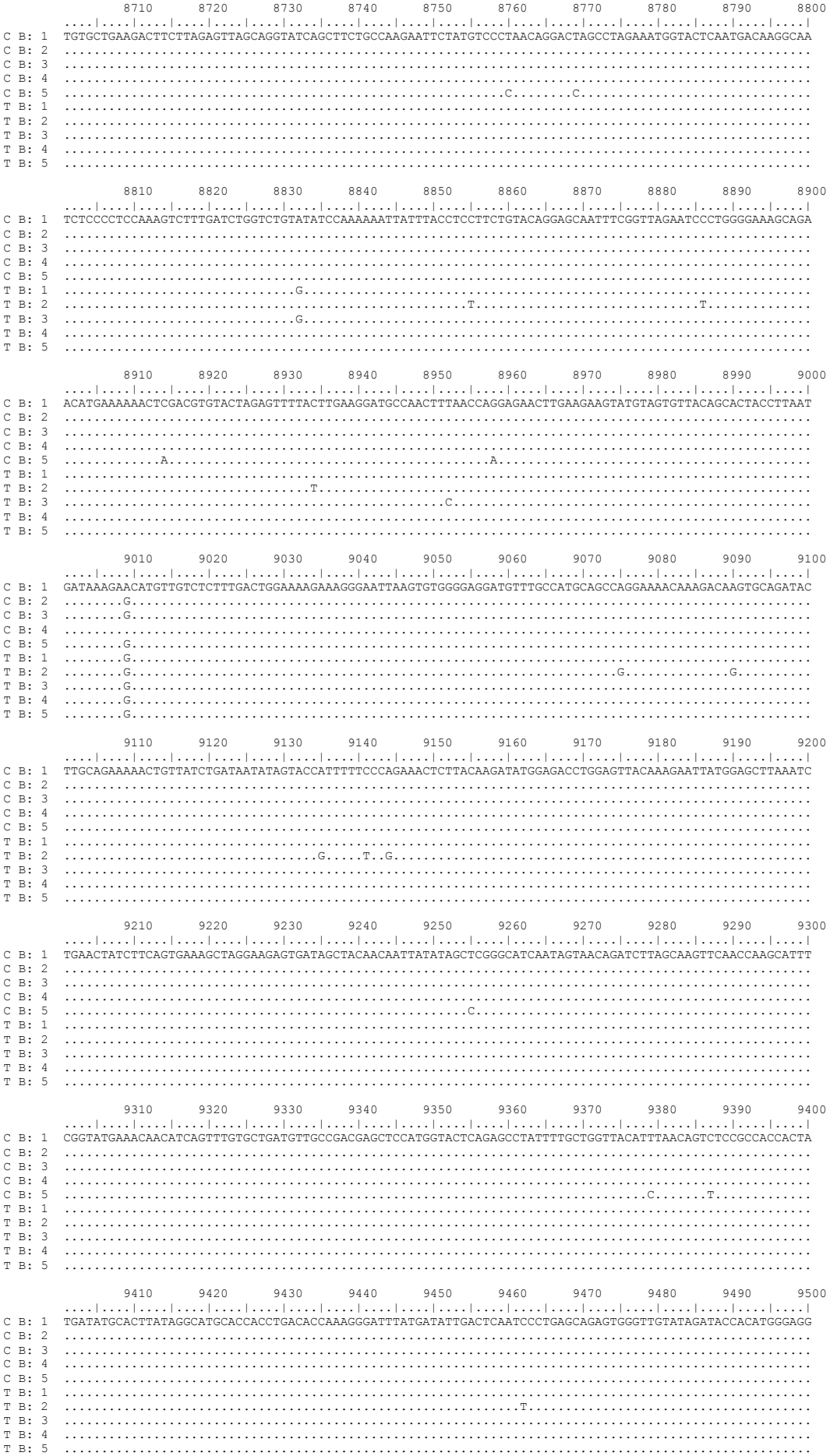

7510 7520 7530 7540 7550 7560 7570 7580 7590 7600
C B: 1 TACACTGCTCATGTTGCAACCACAAGTCCTGTCATTGAGCATCAAAGATTAAAAGGCTTATTTAGTAGTCTAACAATCAGCAAGAACTATCAGTTTGTTG
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5

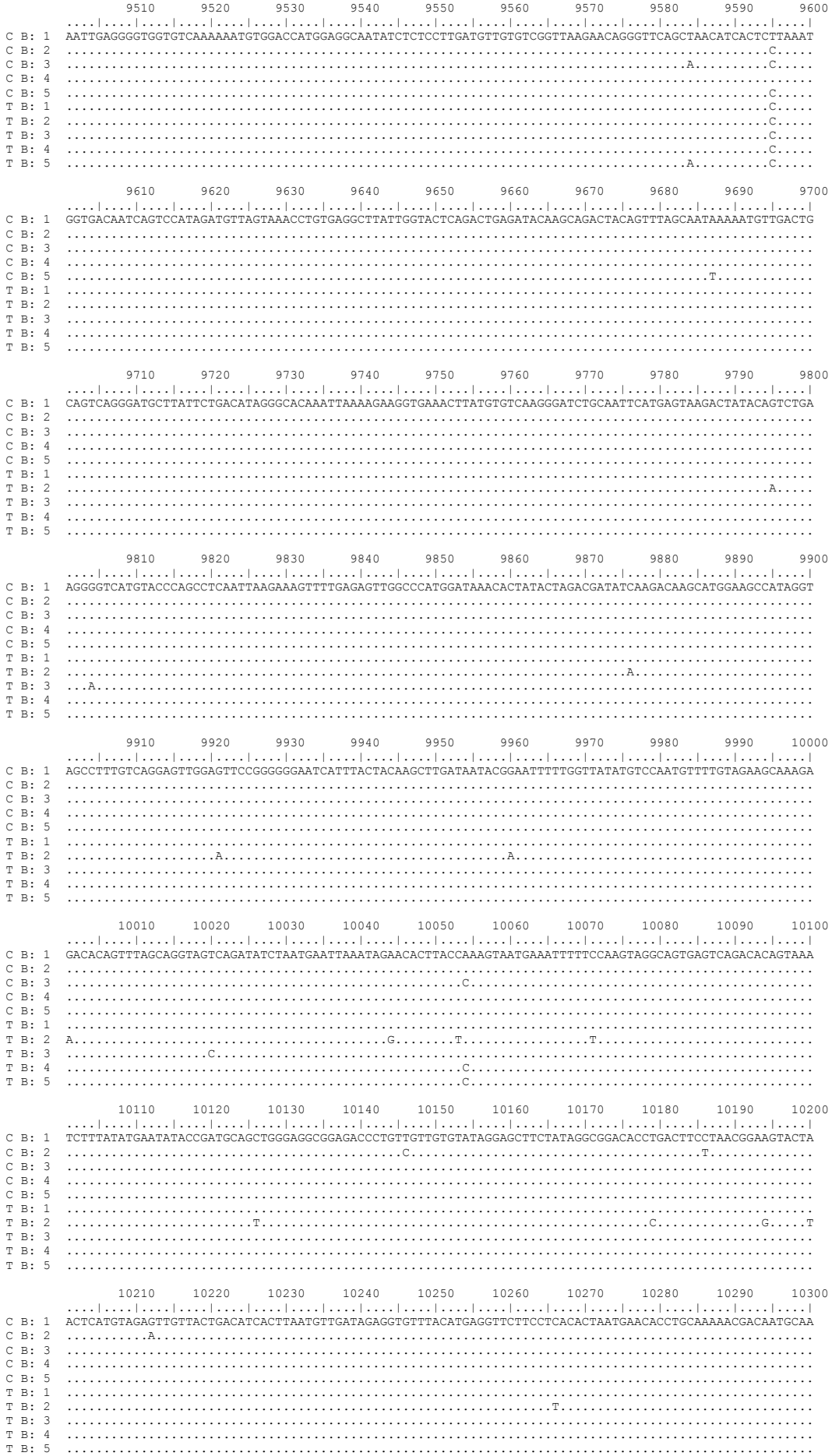

7610 7620 7630 7640 7650 7660 7670 7680 7690 7700
C B: 1 AACCCCTCATGATGCAGAATGAGCTGCTCAAGATCTCTGCCGGTACAAAACTCAAAAAGCTAAAGAAATGGCTAGGCCGAAGCAAAGATATTAGTGAGAT
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5


7710 7720 7730 7740 7750 7760 7770 7780 7790 7800
C B: 1 CAAGCTGAAGATGGTTGCAGACTGGCTCAAACTTGCACATACTCCTGGGAAAGGGAAAGTGATTGACTGTATCCAGGTAGACAACCTACCTGAATGGCTA
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5


7810 7820 7830 7840 7850 7860 7870 7880 7890 7900
C B: 1 GAGCAATGGTTTGATTCATGGTTGATGCTTAATGAACTAGTACAAGCTTATAGGTGCCTGGAAGTGTCACAGACCAGTGCTATCCTCCGTAAAAGCTGTT
C B: 2
C B: 3
C B: 4
C B: 5
T B: 1
T B: 2
T B: 3
T B: 4
T B: 5


7910 7920 7930 7940 7950 7960 7970 7980 7990 8000
C B: 1 GGAATTTCTTCTTTGCTATATCATCATTTGGCTGCATCTTGATAAGCCGGAAAAGTAAAAGGATTTGTTTCTGCACCTATAACCAGTTCTTGACATGGAA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8010 8020 8030 8040 8050 8060 8070 8080 8090 8100
C B: 1 AGATTTGGCACTTAGCAGGTTCAATGCTAACCTGTGTGTCTGGGTTAGCAACTGTTTAAATAGTACTCAAGAAGGACTAGGACTCCGGAGCAAACTCGTT
C B: 2 .....C.....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8110 8120 8130 8140 8150 8160 8170 8180 8190 8200
C B: 1 GGAGAACTCCTAAATCGATTATATCATGAGACTGATGAGTTGTTGAGTGGTACTGGCAATGAAGGATTCTGGATAGTTAAGGAATTCGAGGGATTCATCA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8210 8220 8230 8240 8250 8260 8270 8280 8290 8300
C B: 1 TGAGTGAAATTCTGAGGATAACTGAACATGCACAGTTTAGTGTACGCTTTAGGAACACACTATTAAATGGAATCGTAGAAAAAATTGGGAAAATGCGTAT
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....C.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8310 8320 8330 8340 8350 8360 8370 8380 8390 8400
C B: 1 TGCCTACAGTCAGCGAACTTCTAACACTGCCATAGAAAATCATAGATATCCCGGAGAGCAAAGTTTGTTGGAGAAAACAGGAAGAGTGCTCAAGATTATA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 ....C.....C.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8410 8420 8430 8440 8450 8460 8470 8480 8490 8500
C B: 1 AAGCTACTAGTCCAAAACGATATGTCCAATGCTGCAGAGATGTATTTTATCTTTAGAATATTCGGTCACCCTATGGTAGAAGAAAGGGAGGCCATGGATG
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....A.....
T B: 1 .....C.....
T B: 2 .....C.....G.....
T B: 3 .....C.....
T B: 4 .....
T B: 5 .....
8510 8520 8530 8540 8550 8560 8570 8580 8590 8600
C B: 1 CTGTTCGGGAGAACAGTGAGGCCACTAAAATATTGAGCCTCAGGGCTCTGACAGAGATGAGAGGAGCTTTTATCCTAAGAGTGATCAAAGGTTTTGTAAC
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8610 8620 8630 8640 8650 8660 8670 8680 8690 8700
C B: 1 AAATTACAAGAGGTGGCCAAGAATAAAGAACCCTTCTACATTGAGTGGCAGGTGGCGAATGTATATGAGGGCTAAAACATATCCTAGTCAACTTGAGCTA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....A.....
T B: 1 .....
T B: 2 ......T.....
T B: 3 .....C.....
T B: 4 .....
T B: 5 .....


8710 8720 8730 8740 8750 8760 8770 8780 8790 8800
C B: 1 TGTGCTGAAGACTTCTTAGAGTTAGCAGGTATCAGCTTCTGCCAAGAATTCTATGTCCCTAACAGGACTAGCCTAGAAATGGTACTCAATGACAAGGCAA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....C.....C.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
8810 8820 8830 8840 8850 8860 8870 8880 8890 8900
C B: 1 TCTCCCCTCCAAAGTCTTTGATCTGGTCTGTATATCCAAAAAATTATTTACCTCCTTCTGTACAGGAGCAATTTCGGTTAGAATCCCTGGGGAAAGCAGA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....G.....
T B: 2 .....T.....T.....
T B: 3 .....G.....
T B: 4 .....
T B: 5 .....
8910 8920 8930 8940 8950 8960 8970 8980 8990 9000
C B: 1 ACATGAAAAAACTCGACGTGTACTAGAGTTTTACTTGAAGGATGCCAACTTTAACCAGGAGAACTTGAAGAAGTATGTAGTGTTACAGCACTACCTTAAT
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....A.....A.....
T B: 1 .....
T B: 2 .....T.....
T B: 3 .....C.....
T B: 4 .....
T B: 5 .....
9010 9020 9030 9040 9050 9060 9070 9080 9090 9100
C B: 1 GATAAAGAACATGTTGTCTCTTTGACTGGAAAAGAAAGGGAATTAAGTGTGGGGAGGATGTTTGCCATGCAGCCAGGAAAACAAAGACAAGTGCAGATAC
C B: 2 ....G.....
C B: 3 ....G.....
C B: 4 .....
C B: 5 ....G.....
T B: 1 ....G.....
T B: 2 ........G.....G.....G.....
T B: 3 ....G.....
T B: 4 ....G.....
T B: 5 ....G.....
9110 9120 9130 9140 9150 9160 9170 9180 9190 9200
C B: 1 TTGCAGAAAAACTGTTATCTGATAATATAGTACCATTTTTCCCAGAAACTCTTACAAGATATGGAGACCTGGAGTTACAAAGAATTATGGAGCTTAAATC
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....G.....T..G.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
9210 9220 9230 9240 9250 9260 9270 9280 9290 9300
C B: 1 TGAACTATCTTCAGTGAAAGCTAGGAAGAGTGATAGCTACAACAATTATATAGCTCGGGCATCAATAGTAACAGATCTTAGCAAGTTCAACCAAGCATTT
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....C.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
9310 9320 9330 9340 9350 9360 9370 9380 9390 9400
C B: 1 CGGTATGAAACAACATCAGTTTGTGCTGATGTTGCCGACGAGCTCCATGGTACTCAGAGCCTATTTTGCTGGTTACATTTAACAGTCTCCGCCACCACTA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....C.....T.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
9410 9420 9430 9440 9450 9460 9470 9480 9490 9500
C B: 1 TGATATGCACTTATAGGCATGCACCACCTGACACCAAAGGGATTTATGATATTGACTCAATCCCTGAGCAGAGTGGGTTGTATAGATACCACATGGGAGG
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....T.....
T B: 3 .....
T B: 4 .....
T B: 5 .....


9510 9520 9530 9540 9550 9560 9570 9580 9590 9600
C B: 1 AATTGAGGGGTGGTGTCAAAAAATGTGGACCATGGAGGCAATATCTCTCCTTGATGTTGTGTCGGTTAAGAACAGGGTTCAGCTAACATCACTCTTAAAT
C B: 2 .....C.....
C B: 3 .....A.....C.....
C B: 4 .....
C B: 5 .....C.....
T B: 1 .....C.....
T B: 2 .....C.....
T B: 3 .....C.....
T B: 4 .....C.....
T B: 5 .....A.....C.....
9610 9620 9630 9640 9650 9660 9670 9680 9690 9700
C B: 1 GGTGACAATCAGTCCATAGATGTTAGTAAACCTGTGAGGCTTATTGGTACTCAGACTGAGATACAAGCAGACTACAGTTTAGCAATAAAAATGTTGACTG
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....T.....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....
9710 9720 9730 9740 9750 9760 9770 9780 9790 9800
C B: 1 CAGTCAGGGATGCTTATTCTGACATAGGGCACAAATTAAAAGAAGGTGAAACTTATGTGTCAAGGGATCTGCAATTCATGAGTAAGACTATACAGTCTGA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....A.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
9810 9820 9830 9840 9850 9860 9870 9880 9890 9900
C B: 1 AGGGGTCATGTACCCAGCCTCAATTAAGAAAGTTTTGAGAGTTGGCCCATGGATAAACACTATACTAGACGATATCAAGACAAGCATGGAAGCCATAGGT
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....A.....
T B: 3 ...A.....
T B: 4 .....
T B: 5 .....
9910 9920 9930 9940 9950 9960 9970 9980 9990 10000
C B: 1 AGCCTTTGTCAGGAGTTGGAGTTCCGGGGGGAATCATTTACTACAAGCTTGATAATACGGAATTTTTGGTTATATGTCCAATGTTTTGTAGAAGCAAAGA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....A.....A.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
10010 10020 10030 10040 10050 10060 10070 10080 10090 10100
C B: 1 GACACAGTTTAGCAGGTAGTCAGATATCTAATGAATTAAATAGAACACTTACCAAAGTAATGAAATTTTTCCAAGTAGGCAGTGAGTCAGACACAGTAAA
C B: 2 .....
C B: 3 .....C.....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 A.....G.....T.....T.....
T B: 3 .....C.....
T B: 4 .....C.....
T B: 5 .....C.....
10110 10120 10130 10140 10150 10160 10170 10180 10190 10200
C B: 1 TCTTTATATGAATATACCGATGCAGCTGGGAGGCGGAGACCCTGTTGTTGTGTATAGGAGCTTCTATAGGCGGACACCTGACTTCCTAACGGAAGTACTA
C B: 2 .....C.....T.....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....T.....C.....G.....T
T B: 3 .....
T B: 4 .....
T B: 5 .....
10210 10220 10230 10240 10250 10260 10270 10280 10290 10300
C B: 1 ACTCATGTAGAGTTGTTACTGACATCACTTAATGTTGATAGAGGTGTTTACATGAGGTTCTTCCTCACACTAATGAACACCTGCAAAAACGACAATGCAA
C B: 2 .....A.....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....T.....
T B: 3 .....
T B: 4 .....
T B: 5 .....

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                10310     10320     10330     10340     10350     10360     10370     10380     10390     10400
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  CATTAACTACACTCATGAGAGATCCTCAGGCTATTGGGTCGGAAAGGCAGGCTAAAATTACAAGTGAGATCAATCGTACAGCAGTCACAAGTGTGCTAAG
C B: 2  ....................................................................................................
C B: 3  ....................................................................................................
C B: 4  ....................................................................................................
C B: 5  ..........................................................A.................................A.....
T B: 1  ....................................................................................................
T B: 2  ....................................................................................................
T B: 3  ....................................................................................................
T B: 4  ....................................................................................................
T B: 5  ....................................................................................................

                10410     10420     10430     10440     10450     10460     10470     10480     10490     10500
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  CCTGGCCCCTAACCAGCTTTTTAGTGATAGTGCAGTACATTTTAGCCAGAATGAGGAAGAAATTGGTACTGTTATGCAAGATGTAGGGCCTGTATACCCT
C B: 2  ....................................................................................................
C B: 3  ......T.............................................................................................
C B: 4  ....................................................................................................
C B: 5  ......T.............................................................................................
T B: 1  ....................................................................................................
T B: 2  ....................................................................................................
T B: 3  ....................................................................................................
T B: 4  ......T.............................................................................................
T B: 5  ......T.............................................................................................

                10510     10520     10530     10540     10550     10560     10570     10580     10590     10600
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  CATGGCTTAAGGGTCATATATGAAGCATTCCCATTCCACAAAGCTGAAAAAGTAGTTAACATGATAGCAGGTACCAAATCTATTACAAATATATTGCAAA
C B: 2  ....................................................................................................
C B: 3  ....................................................................................................
C B: 4  ....................................................................................................
C B: 5  ....................................................................................................
T B: 1  ....................................................................................................
T B: 2  ....................................................................................................
T B: 3  ....................................................................................................
T B: 4  ....................................................................................................
T B: 5  ....................................................................................................

                10610     10620     10630     10640     10650     10660     10670     10680     10690     10700
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  GGACATCTGCCATAAGTGGTTTAGACATTGATAGAGCAGTTCATATGATGTTGCTCAATCTAGGACTGTTAGGGAGGATACTAGAGTCAGGACCTGTCAC
C B: 2  ....................................................................................................
C B: 3  .......C............................................................................................
C B: 4  ....................................................................................................
C B: 5  .......C.....................................................................T..........................
T B: 1  ....................................................................................................
T B: 2  ..................................................................G.................................
T B: 3  ....................................................................................................
T B: 4  .......C............................................................................................
T B: 5  .......C............................................................................................

                10710     10720     10730     10740     10750     10760     10770     10780     10790     10800
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  AGACACCATTGAGTTGCGTCACAACAATAGGATACTTTGTTGTCAATTATCAAAGCGCATACGGGAAACATCATGGGACGGAATAGAAATTGTAGGAGTG
C B: 2  ....................................................................................................
C B: 3  ....................................................................................................
C B: 4  ....................................................................................................
C B: 5  ....................................................................................................
T B: 1  ....................................................................................................
T B: 2  ....................................C...............................................................
T B: 3  ....................................................................................................
T B: 4  ....................................................................................................
T B: 5  ....................................................................................................

                10810     10820     10830     10840     10850     10860     10870     10880     10890     10900
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  TCATCCCCTAGTATGTTATCATGCTTGGATATAAACTATGTGACAGCAGCACAGAGGCCAGGAGTATTAATAGAGAAGTTCTCGGCAGAAAAGACTACAA
C B: 2  ....................................................................................................
C B: 3  ....................................................................................................
C B: 4  ....................................................................................................
C B: 5  ....................................................................................................
T B: 1  ....................................................................................................
T B: 2  ..................................................................................A.......G.........
T B: 3  ....................................................................................................
T B: 4  ....................................................................................................
T B: 5  ....................................................................................................

                10910     10920     10930     10940     10950     10960     10970     10980     10990     11000
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  GAGGGAAGAGAGGGCCTAAGGCTCCATGGGTTGGATCTAGCACGCAGGAGAAAAAATTAACAGCAGTGTACAACAGGCAAGCCTTATCAAAAGAGCAGAG
C B: 2  ....................................................................................................
C B: 3  ....................................................................................................
C B: 4  ....................................................................................................
C B: 5  ....................................................................................................
T B: 1  ....................................................................................................
T B: 2  ..................................................................................T.............A..
T B: 3  ....................................................................................................
T B: 4  ....................................................................................................
T B: 5  ....................................................................................................

                11010     11020     11030     11040     11050     11060     11070     11080     11090     11100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C B: 1  AGATCAGTTAGAAACAATAGGTAAGATTAGATGGGTGTACAGAGGGGTAACAGGACTACGGAGACTTTTAGATCTAGTCTGCATGGGAACCTTAGGACTT
C B: 2  ....................................................................................................
C B: 3  ....................................................................................................
C B: 4  ....................................................................................................
C B: 5  ....................................................................................................
T B: 1  ....................................................................................................
T B: 2  ..........................................................A.........................................
T B: 3  ....................................................................................................
T B: 4  ....................................................................................................
T B: 5  ....................................................................................................
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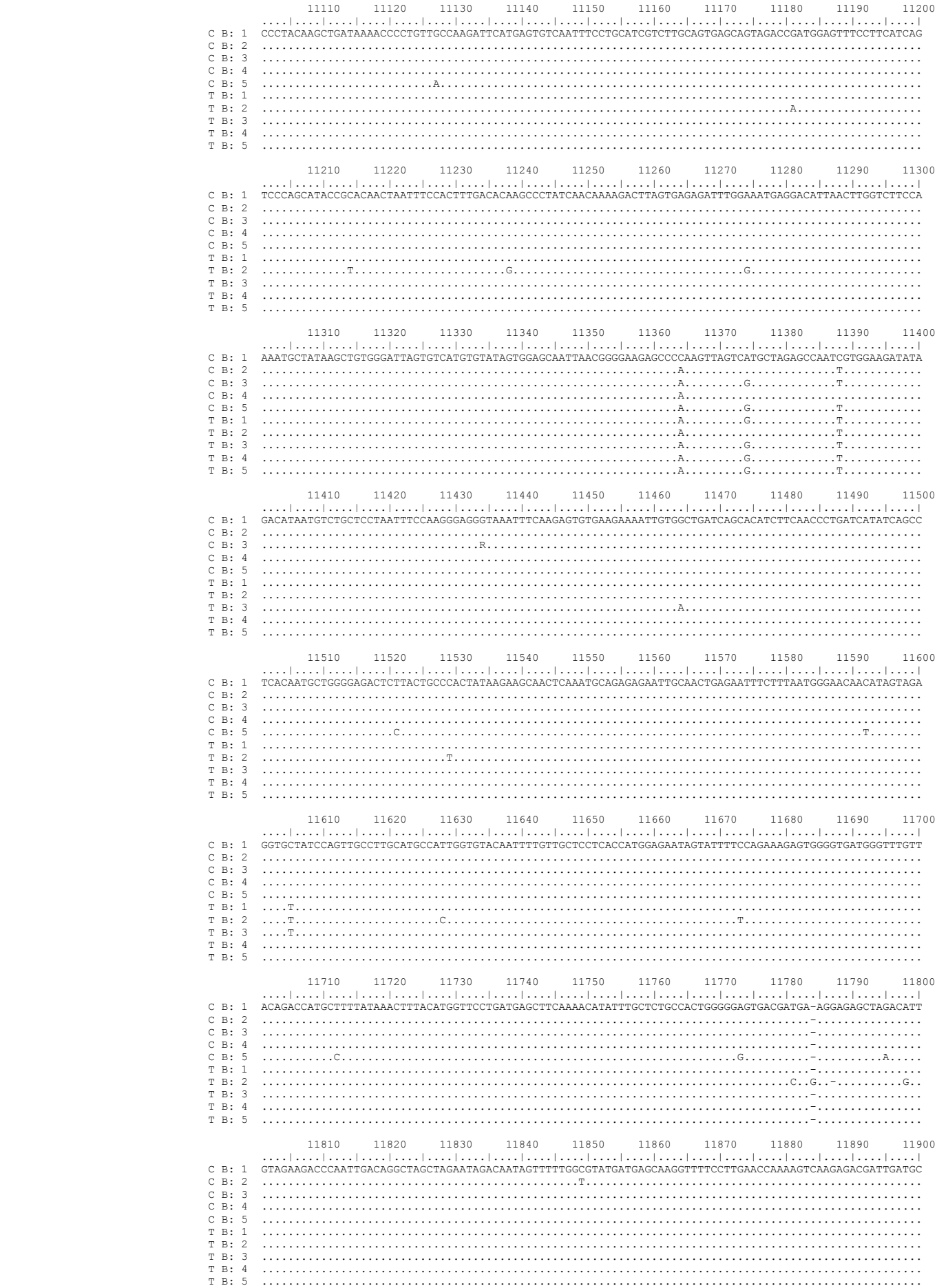
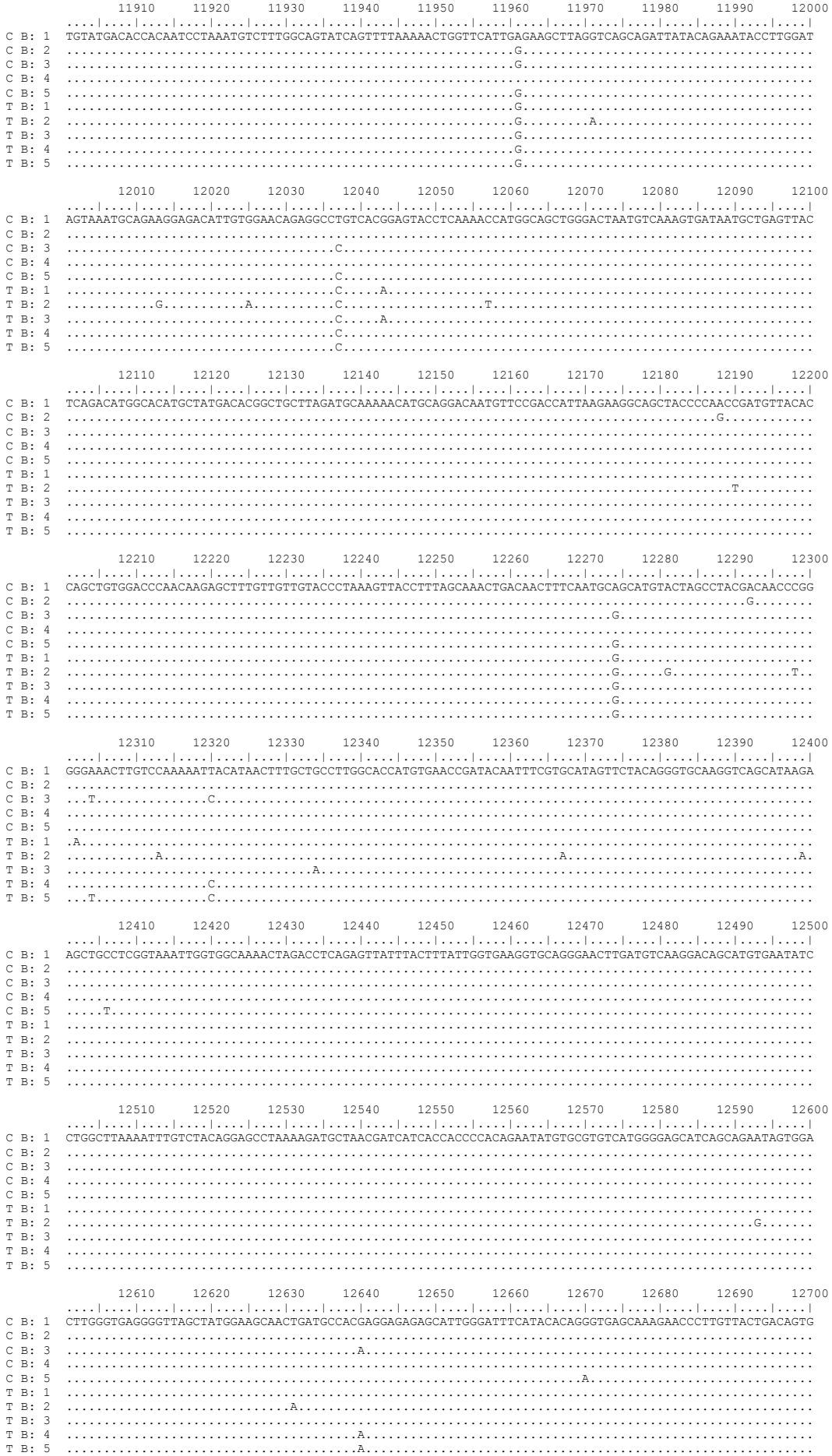
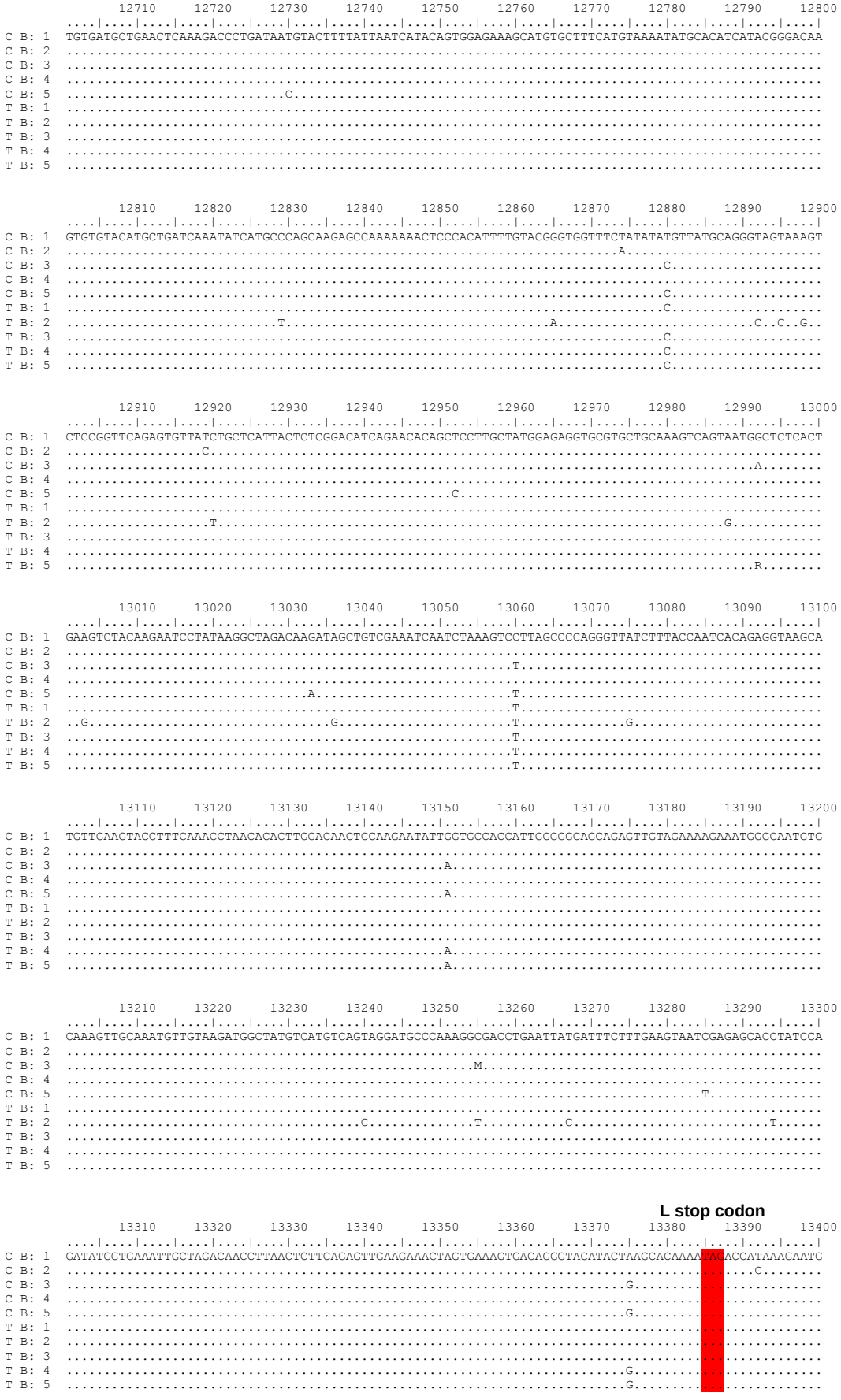

11110 11120 11130 11140 11150 11160 11170 11180 11190 11200
C B: 1 CCCTACAAGCTGATAAAACCCCTGTTGCCAAGATTCATGAGTGTCAATTTCCTGCATCGTCTTGCAGTGAGCAGTAGACCGATGGAGTTTCCTTCATCAG
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....A.....
T B: 1 .....
T B: 2 .....A.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
11210 11220 11230 11240 11250 11260 11270 11280 11290 11300
C B: 1 TCCCAGCATACCGCACAACTAATTTCCACTTTGACACAAGCCCTATCAACAAAAGACTTAGTGAGAGATTTGGAAATGAGGACATTAACTTGGTCTTCCA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....T.....G.....G.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
11310 11320 11330 11340 11350 11360 11370 11380 11390 11400
C B: 1 AAATGCTATAAGCTGTGGGATTAGTGTCATGTGTATAGTGGAGCAATTAACGGGGAAGAGCCCCAAGTTAGTCATGCTAGAGCCAATCGTGGAAGATATA
C B: 2 .....A.....T.....
C B: 3 .....A.....G.....T.....
C B: 4 .....A.....
C B: 5 .....A.....G.....T.....
T B: 1 .....A.....G.....T.....
T B: 2 .....A.....T.....
T B: 3 .....A.....G.....T.....
T B: 4 .....A.....G.....T.....
T B: 5 .....A.....G.....T.....
11410 11420 11430 11440 11450 11460 11470 11480 11490 11500
C B: 1 GACATAATGTCTGCTCCTAATTTCCAAGGGAGGGTAAATTTCAAGAGTGTGAAGAAAATTGTGGCTGATCAGCACATCTTCAACCCTGATCATATCAGCC
C B: 2 .....
C B: 3 .....R.....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....A.....
T B: 4 .....
T B: 5 .....
11510 11520 11530 11540 11550 11560 11570 11580 11590 11600
C B: 1 TCACAATGCTGGGGAGACTCTTACTGCCCACTATAAGAAGCAACTCAAATGCAGAGAGAATTGCAACTGAGAATTTCTTTAATGGGAACAACATAGTAGA
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....C.....T.....
T B: 1 .....
T B: 2 .....T.....
T B: 3 .....
T B: 4 .....
T B: 5 .....
11610 11620 11630 11640 11650 11660 11670 11680 11690 11700
C B: 1 GGTGCTATCCAGTTGCCTTGCATGCCATTGGTGTACAATTTTGTTGCTCCTCACCATGGAGAATAGTATTTTCCAGAAAGAGTGGGGTGATGGGTTTGTT
C B: 2 .....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 ....T.....
T B: 2 ....T.....C.....T.....
T B: 3 ....T.....
T B: 4 .....
T B: 5 .....
11710 11720 11730 11740 11750 11760 11770 11780 11790 11800
C B: 1 ACAGACCATGCTTTTATAAACTTTACATGGTTCCTGATGAGCTTCAAAACATATTTGCTCTGCCACTGGGGGAGTGACGATGA-AGGAGAGCTAGACATT
C B: 2 .....-.....
C B: 3 .....-.....
C B: 4 .....-.....
C B: 5 .....C.....G.....-.....A.....
T B: 1 .....-.....
T B: 2 .....C..G..-.....G..
T B: 3 .....-.....
T B: 4 .....-.....
T B: 5 .....-.....
11810 11820 11830 11840 11850 11860 11870 11880 11890 11900
C B: 1 GTAGAAGACCCAATTGACAGGCTAGCTAGAATAGACAATAGTTTTTGGCGTATGATGAGCAAGGTTTTCCTTGAACCAAAAGTCAAGAGACGATTGATGC
C B: 2 .....T.....
C B: 3 .....
C B: 4 .....
C B: 5 .....
T B: 1 .....
T B: 2 .....
T B: 3 .....
T B: 4 .....
T B: 5 .....


11910 11920 11930 11940 11950 11960 11970 11980 11990 12000
C B: 1 TGTATGACACCACAATCCTAAATGTCTTTGGCAGTATCAGTTTTAAAAACTGGTTCATTGAGAAGCTTAGGTCAGCAGATTATACAGAAATACCTTGGAT
C B: 2 ....G..........
C B: 3 ....G..........
C B: 4 ....
C B: 5 ....G..........
T B: 1 ....G..........
T B: 2 ..........G.........A..........
T B: 3 ....G..........
T B: 4 ....G..........
T B: 5 ....G..........
12010 12020 12030 12040 12050 12060 12070 12080 12090 12100
C B: 1 AGTAAATGCAGAAGGAGACATTGTGGAACAGAGGCCTGTCACGGAGTACCTCAAAACCATGGCAGCTGGGACTAATGTCAAAGTGATAATGCTGAGTTAC
C B: 2 ....
C B: 3 ....C..........
C B: 4 ....
C B: 5 ....C..........
T B: 1 ..........C.....A..........
T B: 2 ...........G..........A..........C..................T..........
T B: 3 ..........C.....A..........
T B: 4 ....C..........
T B: 5 ....C..........
12110 12120 12130 12140 12150 12160 12170 12180 12190 12200
C B: 1 TCAGACATGGCACATGCTATGACACGGCTGCTTAGATGCAAAAACATGCAGGACAATGTTCCGACCATTAAGAAGGCAGCTACCCCAACCGATGTTACAC
C B: 2 ....G..........
C B: 3 ....
C B: 4 ....
C B: 5 ....
T B: 1 ....
T B: 2 ....T..........
T B: 3 ....
T B: 4 ....
T B: 5 ....
12210 12220 12230 12240 12250 12260 12270 12280 12290 12300
C B: 1 CAGCTGTGGACCCAACAAGAGCTTTGTTGTTGTACCCTAAAGTTACCTTTAGCAAACTGACAACTTTCAATGCAGCATGTACTAGCCTACGACAACCCGG
C B: 2 ....G..........
C B: 3 ....G..........
C B: 4 ....
C B: 5 ....G..........
T B: 1 ....G..........
T B: 2 ..........G......G.............T..
T B: 3 ....G..........
T B: 4 ....G..........
T B: 5 ....G..........
12310 12320 12330 12340 12350 12360 12370 12380 12390 12400
C B: 1 GGGAAACTTGTCCAAAAATTACATAACTTTGCTGCCTTGGCACCATGTGAACCGATACAATTTCGTGCATAGTTCTACAGGGTGCAAGGTCAGCATAAGA
C B: 2 ....
C B: 3 ...T............C..........
C B: 4 ....
C B: 5 ....
T B: 1 .A..........
T B: 2 ...........A..........A..........A.
T B: 3 ....A..........
T B: 4 ....C..........
T B: 5 ...T............C..........
12410 12420 12430 12440 12450 12460 12470 12480 12490 12500
C B: 1 AGCTGCCTCGGTAAATTGGTGGCAAAACTAGACCTCAGAGTTATTTACTTTATTGGTGAAGGTGCAGGGAACTTGATGTCAAGGACAGCATGTGAATATC
C B: 2 ....
C B: 3 ....
C B: 4 ....
C B: 5 .....T..........
T B: 1 ....
T B: 2 ....
T B: 3 ....
T B: 4 ....
T B: 5 ....
12510 12520 12530 12540 12550 12560 12570 12580 12590 12600
C B: 1 CTGGCTTAAAATTTGTCTACAGGAGCCTAAAAGATGCTAACGATCATCACCACCCCACAGAATATGTGCGTGTCATGGGGAGCATCAGCAGAATAGTGGA
C B: 2 ....
C B: 3 ....
C B: 4 ....
C B: 5 ....
T B: 1 ....
T B: 2 ....G..........
T B: 3 ....
T B: 4 ....
T B: 5 ....
12610 12620 12630 12640 12650 12660 12670 12680 12690 12700
C B: 1 CTTGGGTGAGGGGTTAGCTATGGAAGCAACTGATGCCACGAGGAGAGAGCATTGGGATTTCATACACAGGGTGAGCAAAGAACCCTTGTTACTGACAGTG
C B: 2 ....
C B: 3 ....A..........
C B: 4 ....
C B: 5 ....A..........
T B: 1 ....
T B: 2 ....A..........
T B: 3 ....
T B: 4 ....A..........
T B: 5 ....A..........


12710 12720 12730 12740 12750 12760 12770 12780 12790 12800
C B: 1 TGTGATGCTGAACTCAAAGACCCTGATAATGTACTTTTATTAATCATACAGTGGAGAAAGCATGTGCTTTCATGTAAAATATGCACATCATACGGGACAA
C B: 2 ....
C B: 3 ....
C B: 4 ....
C B: 5 ....C.....
T B: 1 ....
T B: 2 ....
T B: 3 ....
T B: 4 ....
T B: 5 ....
12810 12820 12830 12840 12850 12860 12870 12880 12890 12900
C B: 1 GTGTGTACATGCTGATCAAATATCATGCCCAGCAAGAGCCAAAAAAACTCCCACATTTTGTACGGGTGGTTTCTATATATGTTATGCAGGGTAGTAAAGT
C B: 2 ....A.....
C B: 3 ....C.....
C B: 4 ....
C B: 5 ....C.....
T B: 1 ....C.....
T B: 2 ..........T.....A.....C..C..G..
T B: 3 ....C.....
T B: 4 ....C.....
T B: 5 ....C.....
12910 12920 12930 12940 12950 12960 12970 12980 12990 13000
C B: 1 CTCCGGTTCAGAGTGTTATCTGCTCATTACTCTCGGACATCAGAACACAGCTCCTTGCTATGGAGAGGTGCGTGCTGCAAAGTCAGTAATGGCTCTCACT
C B: 2 ....C.....
C B: 3 ....A.....
C B: 4 ....
C B: 5 ....C.....
T B: 1 ....
T B: 2 ..........T.....G.....
T B: 3 ....
T B: 4 ....
T B: 5 ....R.....
13010 13020 13030 13040 13050 13060 13070 13080 13090 13100
C B: 1 GAAGTCTACAAGAATCCTATAAGGCTAGACAAGATAGCTGTCGAAATCAATCTAAAGTCCTTAGCCCCAGGGTTATCTTTACCAATCACAGAGGTAAGCA
C B: 2 ....
C B: 3 ....T.....
C B: 4 ....
C B: 5 ..........A.....T.....
T B: 1 ....T.....
T B: 2 ..G.....G.....T.....G.....
T B: 3 ....T.....
T B: 4 ....T.....
T B: 5 ....T.....
13110 13120 13130 13140 13150 13160 13170 13180 13190 13200
C B: 1 TGTTGAAGTACCTTTCAAACCTAACACACTTGGACAACTCCAAGAATATTGGTGCCACCATTGGGGGCAGCAGAGTTGTAGAAAAGAAATGGGCAATGTG
C B: 2 ....
C B: 3 ....A.....
C B: 4 ....
C B: 5 ....A.....
T B: 1 ....
T B: 2 ....
T B: 3 ....
T B: 4 ....A.....
T B: 5 ....A.....
13210 13220 13230 13240 13250 13260 13270 13280 13290 13300
C B: 1 CAAAGTTGCAAATGTTGTAAGATGGCTATGTCATGTCAGTAGGATGCCCAAAGGCGACCTGAATTATGATTTCTTTGAAGTAATCGAGAGCACCTATCCA
C B: 2 ....
C B: 3 ....M.....
C B: 4 ....
C B: 5 ....T.....
T B: 1 ....
T B: 2 ..........C.....T.....C.....T.....
T B: 3 ....
T B: 4 ....
T B: 5 ....
L stop codon
13310 13320 13330 13340 13350 13360 13370 13380 13390 13400
C B: 1 GATATGGTGAAATTGCTAGACAACCTTAACTCTTCAGAGTTGAAGAAACTAGTGAAAGTGACAGGGTACATACTAAGCACAAAATAGACCATAAAGAATG
C B: 2 ....C.....
C B: 3 ....G.....
C B: 4 ....
C B: 5 ....G.....
T B: 1 ....
T B: 2 ....
T B: 3 ....
T B: 4 ....G.....
T B: 5 ....G.....

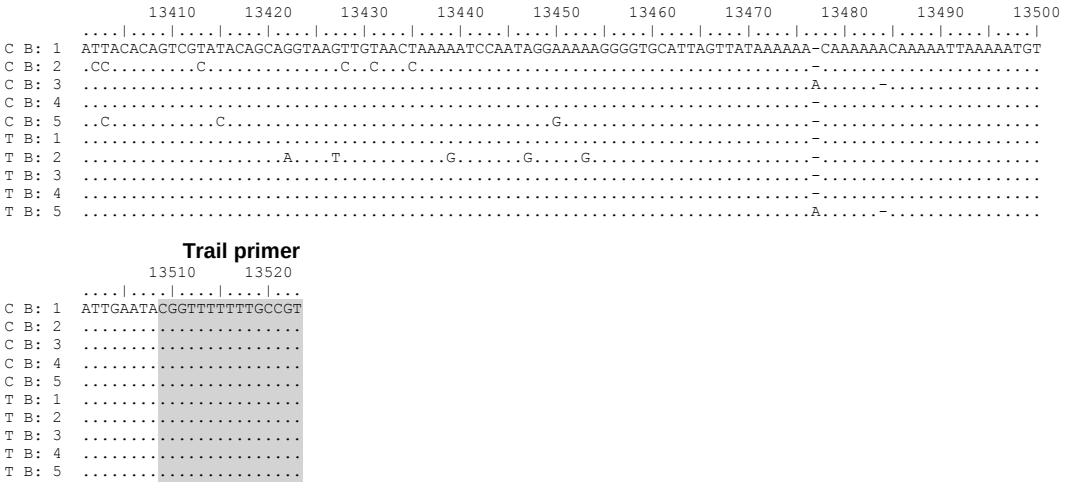


Figure 9. Full nt sequence alignment of subtype B-AMPV isolates. ORF translation start and stop codons are highlighted in green and red respectively. Extreme leader and trailer primer sequence is shown as sequence here was not deduced. Ambiguity code K represents nt G or T, code M represents nt A or C, code R represents nt A or G and code Y represents nt C or T.

(ii) Amino acid sequence comparison.

Compared predicted aa sequences of chicken and turkey derived subtype B AMPVs were highly similar and no regions associated with either host were identified. Unless stated, the aa sequence lengths for each protein were the same as subtype A AMPVs, as mentioned in section 4.3.3 ii, except for the P protein (279aa), the SH protein (175aa) and the G protein (414aa). These are in agreement with previously reported lengths for subtype B AMPV proteins (206).

Within the N protein differences occurred at five aa positions. Due to an ambiguity (code M) in the nt sequence of CB:3, the aa at position 145 could be Lysine (K) as determined for all other isolates, or Threonine (T). The sequence of the P protein was identical for all isolates apart from a single aa difference in isolate TB:2. In the M protein and the F protein differences were identified at four and ten positions respectively. In the M2:1 protein differences occurred between the sequences at three positions. Eight differences were seen in the M2:2 protein, four of which belonged to the chicken-derived vaccine CB: 4. This isolate had an M2:2 protein of 66 aa, seven less than all other isolates, due to two extra nt bases within its open

reading frame, creating an early stop codon. Within the SH protein, differences occurred at 12 positions. Chicken-derived field isolate CB: 3 and turkey-derived vaccine TB: 5 both had an early stop codon in the same position, making them 43 aa shorter than all other isolates in this protein. The G protein was varied, with aa differences seen in 38 positions. The aa at position 103 in the G protein for isolate CB:3 could not be determined as all three nucleotides of the codon had an ambiguity (code Y). Similarly, aa 106 possessed an ambiguity (code Y), therefore coding for either a Cysteine (C) (as found in all other isolates) or an Arginine (R). Isolate TB:5 possessed a nt ambiguity (code K) in aa 158, which could code for either Phenylalanine (F) (as found in all other isolates) or Leucine (L). In the L protein aa differences were identified at 29 positions. Amongst these, isolate CB:3 possessed a nt ambiguity (code R) in aa 1355, therefore coding for either Valine (V) (as found in all other isolates) or Isoleucine (I). Isolate TB:5 possessed a nt ambiguity (code R) at aa 1874, coding for either Threonine (T) as was determined for isolate CB:3, or Alanine (A) which was identified in all other isolates.

Nine aa coding differences were identified between the French chicken-derived field isolate CB:1 and vaccine strain CB:4. Two were found in the N protein, four in the M2:2 protein, which were caused by two extra nt bases in the vaccine sequence, and one in the M, F and G proteins.

Chicken field isolate CB:3 and turkey-derived vaccine strain TB:5 were found to be almost identical despite originating from two different hosts. Possible differences may only exist between the two isolates in the six aa residues that could not be determined due to nt ambiguities. These occurred in isolate CB:3 at aa 145 in the N protein, aa 103 and aa 106 in the G protein and aa1355 in the L protein, and in isolate TB:5 in the G and L proteins at aa158 and aa1874 respectively. The aa at position 103 in isolate CB:3 could not be determined as all 3 nucleotides of the

codon were ambiguous. In the other five positions, one of the two aa possibilities was always present in the other isolate.

Predicted aa differences are shown in table 6 with the corresponding nt positions as they appear in figure 9.

Table 6. aa differences between chicken and turkey-derived AMPV-B isolates. A dash denotes identity with the aa shown for CB:1. An asterisk denotes an undetermined aa due to nt ambiguities.

Gene	aa position	CB: 1	CB: 2	CB: 3	CB: 4	CB: 5	TB: 1	TB: 2	TB: 3	TB: 4	TB: 5	Genome position
N	107	R	-	-	Q	-	-	-	-	-	-	375
	141	A	-	-	-	-	-	-	V	-	-	477
	145	K	-	*	-	-	-	-	-	-	-	489
	284	E	-	-	K	-	-	-	-	-	-	905
	389	K	-	E	-	-	-	-	-	-	E	1220
P	227	A	-	-	-	-	-	T	-	-	-	1933
M	162	A	-	-	-	S	-	-	-	-	-	2603
	179	A	-	S	-	-	-	-	-	-	S	2654
	217	G	-	-	S	-	-	-	-	-	-	2768
	250	V	-	-	-	-	I	-	I	-	-	2867
F	89	I	-	F	-	-	-	-	-	-	F	3213
	149	S	-	-	-	-	-	-	-	T	-	3393
	178	N	-	-	K	-	-	-	-	-	-	3482
	188	R	-	-	-	-	-	K	-	-	-	3511
	253	R	-	-	-	K	-	-	-	-	-	3707
	296	R	-	-	-	-	-	K	-	-	-	3836
	323	K	E	E	-	E	E	E	E	E	E	3916
	499	A	V	V	-	V	V	V	V	V	V	4445
	506	I	-	V	-	-	-	-	-	V	V	4465
	522	S	-	-	-	P	-	-	-	-	-	4513
M2:1	84	E	-	-	-	-	-	D	-	-	-	4846
	182	G	-	-	-	D	-	-	-	-	-	5139
	184	G	S	S	-	S	S	S	S	S	S	5144
M2:2	10	V	-	-	-	M	-	-	-	-	-	5139
	12	A	-	-	-	-	-	V	-	-	-	5146
	15	R	-	-	-	K	-	-	-	-	-	5155
	19	L	-	-	-	S	-	-	-	-	-	5167
	64	E	-	-	L	-	-	-	-	-	-	5301-02
	65	F	-	-	N	-	-	-	-	-	-	5304-06
	66	V	-	-	L	-	-	-	-	-	-	5307-09
	67	A	-	-	Stop	-	-	-	-	-	-	5310-12
SH	27	R	-	-	-	-	-	Q	-	-	-	5471
	84	N	-	-	-	-	-	S	-	-	-	5642
	85	Q	-	-	-	H	-	-	-	-	-	5646
	86	H	-	Y	-	Y	Y	Y	Y	Y	Y	5650
	99	T	-	-	-	-	-	A	-	-	-	5686
	105	N	-	D	-	D	-	-	-	D	D	5704
	112	Q	R	-	-	-	-	-	-	-	-	5726
	113	R	-	-	-	-	-	K	-	-	-	5729
	132	K	-	Stop	-	-	-	-	-	-	Stop	5788
	144	I	-		-	-	-	-	-	V		5821
	157	N	-		-	-	-	S	-	-		5861
	175	N	-		-	-	-	S	-	-		5915

Gene	aa position	CB: 1	CB: 2	CB: 3	CB: 4	CB: 5	TB: 1	TB: 2	TB: 3	TB: 4	TB: 5	Genome position
G	7	I	-	-	-	T	-	-	-	-	-	6044
	20	Q	R	-	-	-	-	-	-	-	-	6083
	31	G	-	R	-	-	-	-	-	-	R	6115
	31	G	-	-	-	-	-	E	-	-	-	6118
	85	D	-	G	-	-	-	-	-	G	G	6279
	87	T	-	-	-	-	-	I	-	-	-	6285
	103	F	L	-	-	-	-	-	-	-	-	6332
	103	F	-	*	-	-	-	-	-	-	-	6332-34
	106	C	-	*	-	-	-	-	-	-	-	6341
	119	R	-	-	-	H	-	-	-	-	-	6381
	146	T	-	-	-	-	-	A	-	-	-	6461
	150	D	-	-	-	G	-	-	-	-	-	6474
	152	N	I	-	-	-	-	-	-	-	-	6480
	158	F	-	-	-	-	-	-	-	-	*	6499
	168	K	-	-	-	-	-	E	-	-	-	6527
	170	N	-	K	-	K	K	K	-	K	K	6535
	174	T	-	-	-	-	-	I	-	-	-	6546
	198	T	-	-	-	-	-	I	-	-	-	6588
	211	G	-	-	-	E	-	-	-	-	-	6657
	221	S	-	-	-	G	-	-	-	-	-	6686
	232	T	-	-	-	-	-	M	-	-	-	6720
	244	Q	-	-	-	K	-	-	-	-	-	6755
	249	G	-	-	-	S	-	-	-	-	-	6770
	250	S	N	-	-	-	-	-	-	-	-	6774
	259	S	-	-	-	N	-	-	-	-	-	6801
	260	H	-	-	-	-	-	Y	-	-	-	6803
	268	S	-	-	-	-	-	R	-	-	-	6829
	269	K	-	-	-	-	-	E	-	-	-	6830
	271	T	-	-	-	-	-	I	-	-	-	6837
	277	T	-	-	-	-	I	-	-	-	-	6855
	282	G	-	E	-	E	-	-	-	E	E	6875
	321	S	-	-	-	G	-	-	-	-	-	6986
	321	S	-	-	-	-	-	N	-	-	-	6987-88
	324	P	-	-	-	-	-	S	-	-	-	6995
	329	K	-	-	N	-	-	-	-	-	-	7013
	334	T	-	-	-	-	-	-	I	-	-	7023
	337	L	-	-	-	P	-	-	-	-	-	7035-36
	339	D	N	-	-	-	-	-	-	-	-	7040
	349	Q	-	-	-	P	-	-	-	-	-	7071
	378	H	-	-	-	Y	-	Y	-	-	-	7157
	382	A	-	-	-	-	S	S	S	-	-	7169
L	62	G	-	-	-	S	-	-	-	-	-	7555
	72	N	S	-	-	-	-	-	-	-	-	7586
	157	V	-	-	-	I	-	-	-	-	-	7840
	312	Y	-	-	-	H	-	-	-	-	-	8305
	426	G	-	-	-	S	-	-	-	-	-	8647
	495	P	-	-	-	-	-	L	-	-	-	8855
	738	L	-	Q	-	-	-	-	-	-	Q	9584
	877	R	-	-	-	-	-	K	-	-	-	10001
	895	K	-	Q	-	-	-	-	-	Q	Q	10054
	1174	K	-	-	-	-	-	R	-	-	-	10892
	1335	M	-	V	-	V	V	-	V	V	V	11374
	1355	V	-	*	-	-	-	-	-	-	-	11434
	1468	S	-	-	-	G	-	-	-	-	-	11773
	1472	G	-	-	-	-	-	R	-	-	-	11785
	1475	D	-	-	-	N	-	-	-	-	-	11794
	1476	I	-	-	-	-	-	V	-	-	-	11797
	1493	R	C	-	-	-	-	-	-	-	-	11848
	1530	E	G	G	-	G	G	G	G	G	G	11961
	1562	T	-	-	-	-	-	I	-	-	-	12057
	1606	T	A	-	-	-	-	-	-	-	-	12188
	1637	T	-	-	-	-	-	A	-	-	-	12281
	1644	G	-	-	-	-	R	-	-	-	-	12302
	1650	Y	-	H	-	-	-	-	-	H	H	12320
	1676	R	-	-	-	-	-	K	-	-	-	12399
	1741	I	-	-	-	-	-	V	-	-	-	12593
	1874	A	-	T	-	-	-	-	-	-	-	12992
	1874	A	-	-	-	-	-	-	-	-	*	12992
	1888	I	-	-	-	-	-	M	-	-	-	13036
	1927	G	-	S	-	S	-	-	-	S	S	13151
	1961	G	-	*	-	-	-	-	-	-	-	13255

4.3.5 Passage of turkey-derived isolates in chicken TOC.

Species specific motifs were not identified in the comparison study, therefore changes which occurred during passage of turkey AMPV's in chicken TOC could not be attributed to a change towards host specificity.

Mutations were identified in both passaged vaccine strains but only in one of the four field strains (table 7). Of the four nt changes in vaccine strain TA:7, three resulted in aa changes. Subtype B vaccine strain TB:5 had two nt mutations within non-coding regions, and one coding and one non-coding mutation in the F ORF. In the field isolates TB:1 had two nt mutations in the L ORF resulting in aa coding changes, and one nt mutation in the M2-SH non-coding region. Isolates TB:2, TB:3 and TB:4 did not incur any nt mutations following ten passages in chicken TOC.

Table 7. Nt and aa changes in turkey-derived AMPV following ten passages in chicken TOC.

Ref	Field or vaccine strain	Gene / region	Nt position	Nt change	aa change
TA:7	Vaccine	M2:1	4637	ggg to gga	-
		SH	5662	ctt to att	L to I
		G	7050	aca to aaa	T to K
		G	7086	aac to Yac *	N to Y or H
TB:1	Field	M2-SH non-coding	5370	a to R*	-
		L	12223	ttt to ttg	F to L
		L	12302	aga to gga	R to G
TB:5	Vaccine	P-M non-coding	2100	a to R*	-
		M-F non-coding	2930	Deletion (a)	-
		F	3568	ttc to ttt	-
		F	4339	ttt to ttg	F to L

*Ambiguity code Y = nucleotide t or c. Code R = nucleotide a or g.

4.4 Discussion

A FL RT-PCR and sequencing protocol was developed for subtype B AMPV's. This, along with an already established protocol for subtype A AMPV's, enabled the identification of the full genome sequence of a range of subtype A and B field strain isolates and commercially available vaccines of chicken and turkey origin, to determine if species specific motifs exist. Such motifs may be of importance in host range which may highlight the need for vaccine species matching.

In general the AMPV subtype B RT-PCR and sequencing protocol, similar to that developed for AMPV subtype A viruses, worked efficiently and allowed the quick determination of FL subtype B AMPV's. On occasions where the sequencing data was poor, PCRs and sequencing had to be repeated to obtain clear sequence data. The method of RNA circularisation used to determine the sequence of the 3' and 5' extremities was un-efficient and time consuming. One possible explanation is that precise ligation of the extreme genome ends, creating a circular molecule, is hindered by an overlap of the complimentary leader and trailer sequences. As a result the leader and trailer sequences were only tentatively deduced for one AMPV subtype A field isolate (TA.1) The 3' leader sequence of this isolate was tentatively determined as ACGAGAAAAAAACGC from ligation 1. In both ligations 2 and 3 the first four bases, ACGA, were absent otherwise the obtained sequence was identical to ligation 1. The 5' sequence deduced from ligation 2 and 3 was CGGTTTTTTTCTCGT and CGGTTTTTTTTTCGT respectively. Sequence of the 5' end from ligation 1 was poor. Different genome start sequences have been reported previously and it has been suggested that these can result from nuclease activity (114) but also that some viruses possibly exist with different genome sequences in their extremities. Here several possible leader and trailer sequences were observed. However due to their complementarity, it is highly likely that the leader and trailer

sequences generated from ligations 1 and 2 respectively, are correct for the extremities of isolate TA:1. These complimentary sequences allow for the formation of the essential genomic panhandle structure and are consistent with those previously reported for AMPV-A (186). Determining the sequence of genome extremities is not straightforward and as a result many methods have been developed. Here, due to its simplicity the RNA circularisation method was chosen. Other methods were not attempted as most time was dedicated to the sequence determination of the rest of the genome. Therefore the extremities were not taken into account in the comparison study.

A conclusive comparison of subtype A chicken and turkey AMPV's could not be performed as only one chicken-derived subtype A AMPV was sourced for the study. However, this isolate possessed 34 nt differences compared to the turkey-derived isolates. This resulted in 11 aa differences in the predicted aa sequence. Further FL sequencing of chicken AMPV isolates would be needed in a bid to determine whether these differences were species specific markers for subtype A chicken AMPV's, or just individual strain differences. Individual sequence differences were observed throughout the turkey-derived subtype A AMPVs, but no motifs suggestive of host specificity were identified. The subtype B chicken and turkey comparison study identified many random individual differences but no sequence motifs specific for either host were seen.

In other observations, it was noted that only eight nt differences existed between UK field strain TA:1 and its vaccine derivative TA:8. Six were responsible for aa coding changes, one in the M protein, three in the F protein and two in the L protein and of these, four were non conservative. One or several of these mutations may be associated with attenuation of the virus, while some may be associated with adaptation to the cell culture system used in the development of the vaccine.

Sequences of turkey-derived vaccine TB:5 and chicken-derived field isolate CB:3 were almost identical. Nucleotide differences were identified in the P ORF at position 2001 and the G ORF at position 6484, but the predicted aa remained the same. Possible differences may exist in the aa positions where nt ambiguities were identified, two of which were in TB:5 and six of which were in CB:3 . Interestingly both isolates possessed an early stop codon at the same position in the SH ORF. In a recent study the full nt sequence of vaccine TB:5 and its progenitor strain were compared (206). The authors also identified the early stop codon in the SH ORF of the vaccine, and found it was not present in the progenitor virus. It had therefore occurred during the adaptation or attenuation process in the development of the vaccine. The close similarity between the two strains sequenced here leads to the suggestion that CB:3 may be a derivative of vaccine TB:5.

The Italian turkey field isolate TB:2 had an altered transcription start sequence for the SH gene and many single nt differences scattered throughout the genome compared to other isolates. This relatively recent strain, isolated in 2004, was shown to cause disease in turkeys that had been vaccinated with a subtype B vaccine. Interestingly, this vaccine offered protection following challenge with an earlier Italian AMPV-B strain isolated in 1987 (TB:2) (39). This example of evolution, giving the virus the capacity to avoid current vaccine induced immunity, means that continuing assessment of current vaccines and field strain viruses is highly important.

Whilst the FL sequences of chicken and turkey derived AMPV isolates were being determined, several turkey-derived AMPV isolates were passaged ten times in chicken TOC. The passaged viruses were then sequenced in full and re-aligned with the original virus sequence. No species specific motifs were identified in the comparison study, therefore it is unknown whether species specific mutations were created during the passage experiment. However, it was interesting to note that

TOC passage produced more mutations in vaccine strains than in field strains. Therefore the vaccine strains, which had been pressured to grown in a foreign cell line in the attenuation process, appeared to be more unstable once back in a host respiratory cell line. It would be interesting to investigate the effects of these mutations *in vivo* with respect to gain or loss of virulence.

As species specific motifs could not be identified in AMPV subtype B viruses, it would be reasonable to assume that vaccines specific to a particular species are not a requirement. Given the close relationship between subtypes A and B it is likely that the same would have been true for subtype A. However, the sequence of more subtype A chicken-derived isolates would be needed before this could be fully justified.

Chapter 5

Avian metapneumovirus M2:2 protein inhibits replication in Vero cells: Modification facilitates live vaccine development.

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Chapter 5

Avian metapneumovirus M2:2 protein inhibits replication in Vero cells: Modification facilitates live vaccine development.

5.1 Introduction

Shortly after the emergence of AMPV, two major commercially available subtype A live vaccines were produced from a highly virulent AMPV field virus, UK #8544 (ref TA:5 in Chapter 4). One of these vaccines, named Vaccine 1 in this study (ref TA:6 in Chapter 4), was produced at Liverpool by the adaptation and passage of #8544 in Vero cells (165, 234, 235). During the early stages of vaccine development, strain #8544 had been shown to grow readily in avian tracheal tissues and avian fibroblasts but not Vero cells. Vero cell adaptation was eventually achieved following a lengthy adaptation to CEL cells involving multiple centrifugations and trypsin treatments. After 17 passages in Vero cells the virus did not produce disease in one day old turkeys yet conferred full protection three weeks later (234). Sequencing revealed nine nt mutations that arose as a result of adaption or attenuation during the development of Vaccine 1 from field strain #8544 (36). Four of these mutations altered coded amino acids (genome positions 3553, 3825, 5055 and 5140), three were synonymous substitutions (genome positions 6358, 10,022 and 11,624) and a further two were identified in non-coding regions (genome positions 2941 and 5929). This vaccine has since been shown to be unstable and its reversion to virulence has been associated with disease in the field (36).

As a starting point in the quest to develop a vaccine with improved stability a number of these mutations were precisely introduced into the sequence of the parental #8544 genome sequence using an AMPV subtype A RG system. The

principal aim was to identify which individual mutation or combination of mutations permitted growth in Vero cells.

The AMPV subtype-A RG system developed at Liverpool (164) utilizes Vero cells infected with a T7 recombinant fowl pox virus which produces T7 polymerase in the cells. These cells are then transfected with T7 driven cDNA plasmids encoding the FL genome and the proteins of the AMPV ribonuclear protein complex (RNP) (N, P, M2 and L). Following T7 transcription of these plasmids and downstream expression, components assemble at the cell membrane and virus can be rescued. An essential factor in this system is that the virus can replicate in Vero cells.

It was known that a FL DNA copy of Vaccine 1 could be recovered in the Liverpool RG system (29). The sequence of this FL copy was modified to generate a FL DNA copy of #8544. The resulting construct was named p#8544. As expected the virus corresponding to this sequence could not be recovered in the Vero cell based RG system.

To determine which mutations were permitting replication of Vaccine 1 in Vero cells the FL DNA copy p#8544 was manipulated by SDM, using blunt ended RT-PCR products or complimentary primer pairs, to re-introduce Vaccine 1 sequence substitutions.

It was found that a single non-conservative base substitution within the M2 second reading frame was enabling growth in Vero cell culture. Computer software predicted that glycosylation changes were unlikely to be involved. The negation of M2.2 translation by start codon corruption led to an identical outcome. Sequencing of other AMPV subtype A M2.2 genes suggest possible broader application.

5.2 Materials and methods

5.2.1 Generation of #8544 FL genome copies and its derivatives.

Cloned FL genome copies were all developed from a FL DNA copy of Vaccine 1 using SDM (3.7). RT, performed using antigenomic primers (3.2.2), and high fidelity pfu PCRs (3.3.1) were performed to generate blunt-ended amplicons. These were used to introduce several substitutions in one SDM reaction. Complimentary primer pairs were also used in SDM to generate FL copies containing single nt base substitutions. All products generated by SDM were visualised on 0.8% agarose gels (3.4) and treated with *Dpn1* prior to *E.coli* transformation (3.8). Colonies were grown on agar containing kanamycin antibiotic and screened by PCR (3.3.2) then sequenced (3.5). Colonies containing plasmids with the required sequence were grown in liquid culture (3.8i), from which the plasmids were then purified (3.9). Final clones used for virus recovery by RG were sequenced in their entirety using a previously developed subtype A RT-PCR and sequencing system.

The sequence differences between the #8544 FL copy (p#8544), Vaccine 1, and seven other p#8544 derivatives are shown in table 8. Details on their construction are addressed in specific paragraphs (5.2.1 i to iv). Details of primers used are shown in table 9.

Table 8. Sequence differences (highlighted) between field strain #8544, the #8544 derived Vaccine 1 and derivative mutants.

Position[a]	Region[b]	#8544[i]		Vaccine 1		p#8544 vac 4.87-6.37		p#8544 vac 5055		p#8544 vac 5140		p#8544 vac 5929		p#8544 vac 6358		p#8544 no M2:2 AUG 1		p#8544 no M2:2 AUG 1-2	
		RNA[c]	aa[d]	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa
2941	F_{TS}[e]	U	none	A	none	U	none	U	none	U	none	U	none	U	none	U	none	U	none
3553	F	U	V	C	A	U	V	U	V	U	V	U	V	U	V	U	V	U	V
3825	F	G	E	A	K	G	E	G	E	G	E	G	E	G	E	G	E	G	E
5055	M2:1	A	K	G	R	G	R	G	R	A	K	A	K	A	K	A	K	A	K
5104	M2:1	U	N	U	N	U	N	U	N	U	N	U	N	U	N	C	N	C	N
5104	M2:2	U	SC1[f]	U	SC1	U	SC1	U	SC1	U	SC1	U	SC1	U	SC1	C	no SC[h]	C	no SC
5140	M2:1	U	N	C	N	C	N	U	N	C	N	U	N	U	N	U	N	U	N
5140	M2:2	U	I	C	T	C	T	U	I	C	T	U	I	U	I	U	I	U	I
5169	M2:1	A	none	A	none	A	none	A	none	A	none	A	none	A	none	A	none	U	none
5169	M2:2	A	SC2[g]	A	SC2	A	SC2	A	SC2	A	SC2	A	SC2	A	SC2	A	SC2	U	no SC
5929	SH-G	A	none	G	none	G	none	A	none	A	none	G	none	A	none	A	none	A	none
6358	G	U	F	C	F	C	F	U	F	U	F	U	F	C	F	U	F	U	F
10022	L	U	V	G	V	U	V	U	V	U	V	U	V	U	V	U	V	U	V
11624	L	U	Y	C	Y	U	Y	U	Y	U	Y	U	Y	U	Y	U	Y	U	Y

[a] Nucleotide position (antigenome 5' to 3').

[b] Region within genome.

[c] Nucleotide identity at genome position.

[d] Predicted amino acid coded.

[e] Transcription start.

[f] M2:2 1st start codon.

[g] M2:2 2nd start codon.

[h] M2:2 1st and 2nd start codons corrupted.

[i] Virus grew in TOC but not Vero cells.

Table 9. Sequences of primers used for RT-PCR and SDM reactions. Nucleotide position within the genome denotes the binding site at the 5' end of the primer.

Primer Name	Sequence 5' to 3'	Nt position within genome
M8+	gaagctgcaataagtggggaag	2649
M2 mid for	ccagagattcaatgcttgaagaccc	4923
Vac 5055 +	gagaaaaactcaaaaggcttgccaagatcatac	5040
Vac 5055 neg	gtatgatcttggcaagccttttgagtttttctc	5040
M2:2 5104 +	tgacaacgccagtggtaataccc	5098
M2:2 5104 neg	gggtattaccactggcgttgtca	5098
Vac 5140 +	gaagggtgacagcaacaattaagtgcaatgcac	5125
Vac 5140 neg	gtgcattgcacttaattgttgctgtcacccttc	5125
M2:2 5169 +	caatgcacttggattgtgtttggttcg	5151
M2:2 5169 neg	cgaaccaaacacaatccaagtgcattg	5151
Vac 5929 +	gacaacactagtgccaaatgataggcaacag	5910
Vac 5929 neg	ctgttgcctatcatttggcactagtgttgtc	5910
Vac 6358 +	ctgtactgggtggtttcgattgtatgggcttattgg	6342
Vac 6358 neg	ccaataagcccatacaatcgaaaccacccagtacag	6342
G2-A ext	gcccatacaatcaaaaccacccag	6347
G6-A ext	gattatctcgctgacaaattggtcctg	6390
L22 +	ggttagcagatgagagcagc	9943
L9 neg	caccttgctcatcatccgccaa	11,714

(i) p#8544

An exact DNA copy of virulent field strain #8544 was constructed in two stages by SDM modification of a FL DNA copy of Vaccine 1 using two high fidelity blunt ended #8544 RT-PCR amplicons as mega primers. Viral RNA was extracted from field strain #8544 (3.2.1). The two high fidelity RT-PCR products were then generated using primers M8+ and G6-A ext (table 9) corresponding to genome positions 2949 and 6416 respectively (amplicon 1), and primers L22+ and L9 neg (table 9) corresponding to genome positions 9945 and 11,736 respectively (amplicon 2). The cycle listed in section 3.3.1 was used in both cases with the exception that step 7 was reduced to 15 cycles to reduce copying errors. Amplified products were analysed on a 1.5% agarose gel (3.4) then treated with EXO to remove primer sequences (appendix). Amplicon 1 was then utilised as a mega primer in an SDM reaction with the FL DNA clone of vaccine 1 (3.7). The resulting SDM product was then used to generate a purified plasmid clone as described in section 5.2.1. The whole process was then repeated using this clone with amplicon 2 to produce a plasmid clone containing the exact sequence of the field strain #8544. The plasmid clone was named p#8544.

(ii) p#8544 $_{\text{vac 4.87-6.37}}$

High fidelity amplicons, using primers M2 mid for and G2-A ext (table 9), were generated from Vaccine 1 RNA between genome positions 4874 and 6371. The cycle described in 3.3.1 was used with the exception that step 7 was reduced to 15 cycles. The product was analysed on a 1.5% agarose gel (3.4), treated with EXO to remove primer sequences (appendix) and then used as a mega primer in SDM on p#8544 to re-establish Vaccine 1 sequence at genome positions 5055, 5140, 5929 and 6358. The resulting plasmid was named p#8544 $_{\text{vac 4.87-6.37}}$.

(iii) p#8544 $_{vac\ 5055}$, p#8544 $_{vac\ 5140}$, p#8544 $_{vac\ 5929}$ and p#8544 $_{vac\ 6358}$

Four FL DNA copies were developed from p#8544, each with a single Vaccine 1 sequence substitution at genome position 5055, 5140, 5929 or 6358. Complimentary primer pairs were employed in primer SDM with each primer pair containing one of the required substitutions (Vac 5055 + and Vac 5055 -, Vac 5140+ and Vac 5140 neg, Vac 5929+ and Vac 5929 neg, Vac 6358+ and Vac 6358 neg)(table9). FL copies were named p#8544 $_{vac\ 5055}$, p#8544 $_{vac\ 5140}$, p#8544 $_{vac\ 5929}$ and p#8544 $_{vac\ 6358}$.

(iv) p#8544 $_{no\ M2:2\ AUG\ 1}$ and p#8544 $_{no\ M2:2\ AUG\ 1+2}$

Two FL genome copies were generated with corrupted M2:2 open reading frame start codons. The start codon at genome position 5104-5106 was converted, from AUG to ACG, by SDM with p#8544 and primer pairs M2:2 5104+ and M2:2 5104 neg (table9). The resulting plasmid was named p#8544 $_{no\ m2:2\ AUG\ 1}$. A further potential downstream start codon within the same frame, at genome position 5169-5171, was then converted from AUG to UUG using primer pairs M2:2 5169 + and M2:2 5169 neg (table9). The resulting plasmid was named p#8544 $_{no\ M2:2\ AUG\ 1+2}$. The aa sequence of the M2:1 reading frame remained unaltered.

5.2.2 Virus recovery from FL plasmids by reverse genetics

FL DNA copies were transfected, together with other components of the AMPV RG system, as previously described (164). In brief, Vero cells were infected with a fowlpox recombinant virus expressing T7 polymerase. These were then transfected with the FL DNA copies and support plasmids, which contained an upstream T7 promoter transcribing the N, P, M2 and L genes. In all cases 160 ng of the cloned FL copy was transfected with the other RG system components. Cells were observed daily for signs of CPE using low power microscopy. If virus was not recovered, the rescue attempt was repeated both identically and with variations of

the plasmid concentration. To ensure the system was functioning efficiently, a standardised control transfection was performed during each rescue attempt using an SH deletion virus which produces a very clear giant syncytial formation (30). Because any cytopathic effect (CPE) is generally obscured by the effects of the transfection agent (Lipofectamine 2000, Invitrogen), all the transfected cells were freeze thawed after 7 days and used to inoculate further Vero cell monolayers.

5.2.3 Detection of recovered virus

The presence of CPE was investigated daily from the first Vero cell passage onward. If virus could not be detected by any CPE, two further Vero cell passages were made. If virus remained undetectable by CPE in the third passage, evidence of virus recovery was attempted using an established RT-PCR routinely used for sensitive detection of the AMPV G gene in diagnostic field samples (41).

5.2.4 Sequencing of subtype A AMPV M2 genes

To compare M2:2 sequences of different viruses, subtype A AMPV's with a known passage history were sequenced. One group of viruses had been isolated and passaged in chick embryo TOC, sometimes in combination with passage in other chicken primary cells, but none of this group had been adapted to Vero cells. This group comprised chicken isolate CP1 (127), turkey isolate UK3B (157), a second #8544 derived subtype A live vaccine (Vaccine 2), and an obsolete vaccine derived from UK3B (Vaccine 3) (69). A second group, which had been adapted to Vero cells, consisted of Vaccine 1 and LAH A isolated in Germany (164). The M2 genes were sequenced using previously described methods (167) and the predicted aa sequence of the M2:2 reading frames derived.

5.2.5 Prediction of M2:2 glycosylation

NetOglyc 3.1 analysis (130) was performed on predicted M2.2 sequences to investigate potential for O linked glycosylation of S and T amino acids.

5.3 Results

5.3.1 FL clones used in the RG system

PCR and primer SDMs were used successfully in the exchange of sequence between Vaccine 1 and p#8544. All FL clones which were used in attempted virus rescues were sequenced in their entirety and found to be as designed.

5.3.2 Evidence of virus following transfection of FL DNA copies.

(i) p#8544

CPE was not detected in the FL DNA copy of p#8544, in the transfected Vero cells nor the subsequent two passages, after careful daily observations over a seven day period. A repeat transfection was performed, and variations were made to the concentrations of the plasmid components of the RG system, with the same negative outcome. RT-PCR performed within the G gene did not detect evidence of the viral genome.

(ii) p#8544 derivatives

For the FL copy p#8544 $_{vac\ 4.87\text{-}6.37}$, CPE was observed following one passage of transfected cells which was similar to that caused by vaccine 1. This prompted the construction of clones containing individual substitutions within this region. For FL copies p#8544 $_{vac\ 5055}$, p#8544 $_{vac\ 5029}$ and p#8544 $_{vac\ 6358}$ CPE was not observed in the transfected cells nor the subsequent two passages. Similar to p#8544, variations to the plasmid concentrations of the RG system did not change the result and the viral genome could not be detected by RT-PCR's in the G gene. FL copy p#8544 $_{vac}$

$_{5140}$ produced CPE comparable to that of Vaccine 1 and p#8544 $_{vac\ 4.87\text{-}6.37}$ after one passage of the RG transfection. The M2:2 corrupted FL copies, p#8544 $_{no\ M2:2\ AUG\ 1}$ and p#8544 $_{no\ M2:2\ AUG\ 1\ +\ 2}$, also gave CPE similar to that caused by vaccine 1 after one passage of the RG transfection. Examples of CPE observed in Vero cells are shown in figure 10.

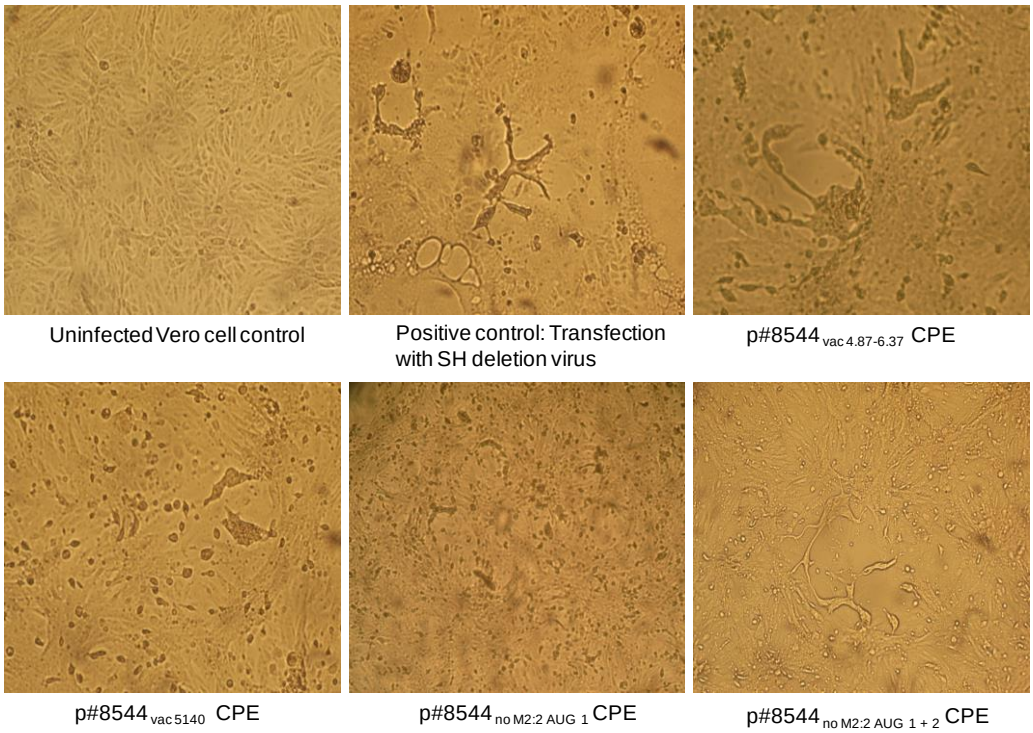

Figure10. CPE produced in Vero cells following transfections with p#8544$_{vac4.87\text{-}6.37}$, p#8544$_{vac5140}$, p#8544$_{no\ M2:2\ AUG\ 1}$ and p#8544$_{no\ M2:2\ AUG\ 1\ +\ 2}$. An example of uninfected Vero cells and the transfection positive control (SH deletion virus) are shown. Magnification x 200.

5.3.3 Sequencing of subtype A AMPV M2 genes

The predicted aa sequences of the M2:2 reading frames are shown in figure 11. Viruses which had not been grown in Vero cells were identical in the M2:2 protein with the exception of amino acid 25. At this position either an Arginine (R) or Lysine (K) (conserved properties) amino acid was observed. However, the two Vero cell grown viruses had predicted non-similar aa substitutions. Vaccine 1 possessed a polar Threonine (T) at residue 12, which had substituted a hydrophobic Isoleucine (I)

in the progenitor strain #8544. Virus LAH A had an aliphatic Leucine (L) substitution at residue 62 compared with the consensus aromatic aa Phenylalanine (F).

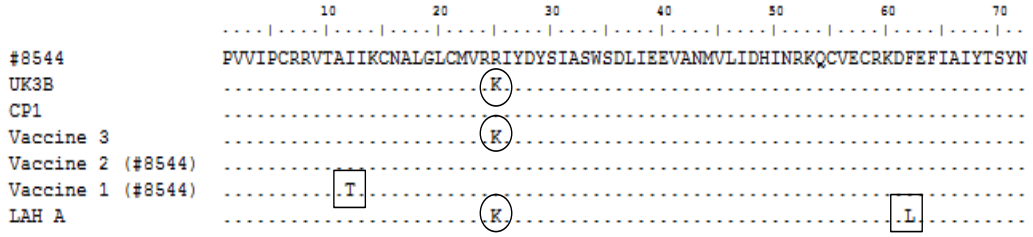

Figure11. M2:2 predicted aa sequences of 7 subtype A AMPVs with known passage history. Square boxes denote non-similar aa substitutions. Circles denote substitutions of similar amino acids.

5.3.4 Prediction of M2:2 glycosylation

The T residue present at residue 12 in Vaccine 1 was not found to be glycosylated by the prediction software NetOglyc 3.1, nor were any of the other four S or two T residues in the protein.

5.4 Discussion

A FL copy of virulent field strain #8544 was developed to enable preliminary studies concerned with making an #8544 based modified vaccine. This was achieved via the conversion of a FL copy of its vaccine derivative (Vaccine 1) by SDM with high fidelity RT-PCR products. Whilst it was known the FL copy of Vaccine 1 could be rescued in the Vero cell based RG system (29) it was found that the FL copy of #8544 could not. Field virus #8544 had previously undergone a lengthy adaptation to Vero cells in the development of Vaccine 1, and the adaptation and attenuation process had led to the nine nt substitutions shown in table 8. Systematic substitution of Vaccine 1 sequence was used to investigate which #8544 sequence was preventing virus recovery by RG and growth in Vero cells.

Of the nine substitutions in Vaccine 1, three sequences changes within the F gene were not considered. In a previous study (168) the F gene of #8544 within a German A type virus (LAH) could be recovered in the RG system suggesting that the #8544 F gene did not prevent replication in Vero cells. Two further nt substitutions in the L gene were not considered to be highly influential as the aa remained unchanged. Vaccine 1 substitutions firstly introduced between genome positions 4874 and 6371 in p#8544 $_{\text{vac 4.87-6.37}}$ led to virus being recovered, with CPE similar to that of Vaccine 1. This implied that any of the differences between #8544 and Vaccine 1 in that region, comprising substitutions at genome positions 5055, 5140, 5929 or 6358, may have been permissive of growth in Vero cells. The coding mutations at 5055 and 5140 were considered likely to have the greatest potential influence. That said the non-coding mutation occurring at position 5929, within the SH-G un-translated region, could not be discounted as mutations in the same region in candidate vaccines highly related to Vaccine 1 had been shown to greatly influence the level of conferred protection (167). The mutation at 6358 was not considered to be highly influential as the predicted aa remained unchanged. Individual Vaccine 1 nt sequence substitutions at genome positions 5055, 5929 and 6358 did not lead to virus recovery by RG, however the substitution at position 5140 led to clear CPE, similar to that of Vaccine 1, after one passage in Vero cells. The predicted effect of the coding change at position 5140 was to leave M2:1 unaffected, but cause a Threonine (T) for Isoleucine (I) substitution in M2:2. This hydrophobic to polar conversion could potentially lead to a significant change in protein properties. It was unclear whether the mutation had modified the M2:2 to one more suitable for growth in Vero cells, or alternatively growth in Vero cells became possible because a functional M2:2 was no longer present. It was hypothesised that T at residue 12 might be O link glycosylated and lead to M2:2 changes facilitating Vero cell growth. However NetOglyc prediction software indicated that neither this residue, nor any of the other S and T residues in the protein were likely to be glycosylated. This led to

the hypothesis that the substitution at genome position 5140 disrupted the function of the M2:2 gene which in turn facilitated growth in Vero cells. To explore this situation further, the FL #8544 copy was modified to corrupt either the M2:2 first (p#8544 $_{\text{no M2:2 AUG 1}}$), or first and second (p#8544 $_{\text{no M2:2 AUG 1+2}}$), AUG start codons while leaving the M2:1 predicted aa code unaltered. Both these FL derivatives readily yielded virus in the RG system, producing CPE similar to that of Vaccine 1 and the p#8544 derivatives. These findings showed that an I-T mutation at position 5140 in the sequence of #8544 permitted growth in Vero cells. As the viruses in which the M2:2 gene was ablated produced the same outcome it is highly likely that the T aa at position 5140 altered the normal function of the M2:2 protein.

Chapter 6

Developing a full length avian metapneumovirus subtype B infectious clone.

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Chapter 6

Developing a full length avian metapneumovirus subtype B infectious clone.

6.1 Introduction

An AMPV subtype A RG system, developed at Liverpool, has been enabling studies into aspects of virulence and protection, and research towards the development of more stable modified subtype A vaccines (29, 82, 146, 164). A similar RG system developed in the USA exists for AMPV subtype C (98) and for many other members of the Paramyoxviridae family (114, 155). These systems allow the generation of virus from cloned FL genome cDNA copies. As yet a system does not exist for AMPV subtype B, although this virus is regularly detected in the field and may be the more dominant subtype (11, 48, 123, 161, 229). It has also been shown to be more invasive and to persist for longer in broilers compared to subtype A (8, 77). In addition, on occasions subtype B vaccines have failed to offer protection against both homologous and heterologous challenge (10, 39, 77, 190) and recently has been shown to revert to virulence (44). A RG system for subtype B AMPV would allow studies into improved modified vaccines for this increasingly important subtype.

A functional subtype B RG system requires cDNA plasmids encoding a FL copy of the subtype B genome and the RNP proteins, N, P, M2 and L. A previous project, aiming to develop an AMPV-B RG system (82) had successfully cloned large regions of subtype B sequence although it had not been possible to obtain a FL cDNA copy in a single plasmid. During that project attempts were initially made to convert a subtype A FL cDNA clone, used in the AMPV-A RG system, to that of subtype B sequence using SDM. Whilst some regions of sequence were converted

to subtype B, many cloning difficulties including SDM failure, poor or no colony growth following transformation of *E.coli,* and deletions in cloned constructs were encountered. The problems increased with each added region of subtype B sequence until further cloning became impossible. Intolerance to certain areas of viral sequence was thought to be the cause resulting in bacterial instability. A plasmid containing sequence from 0-12.1 kb of the subtype B sequence was constructed. Attempts to insert sequence to 13.5 kb to complete the FL did not succeed. In addition genes encoding the support proteins N, P and M2 had also been cloned in full but a complete L gene clone was not obtained. Finally during the course of the study several other plasmids containing varying regions of subtype B sequence had been generated.

In the present study, the plasmid containing AMPV-B sequence 0 to 12.1 kb (named clone pB12.1) and another containing AMPV-B sequence from 12.1 kb to 13.5 kb (named clone pB12-13), were used to generate PCR amplicons in an attempt to generate FL sequence by overlap PCRs. Further attempts were also made to complete clone pB12.1 using varying sized PCR amplicons with standard cut and ligation techniques. Difficulties faced in the previous study were re-encountered. The lack of progression with the current plasmid clones led to a decision to use a different plasmid (pSMART-LC (Kan), Lucigen) specifically designed for the cloning of toxic genes. This low-copy plasmid possessed strong transcription terminators flanking the cloning site which stabilizes otherwise toxic inserts. Previously constructed partial subtype B plasmids were again utilised for the amplification of subtype B sequence, which was ligated with the pSMART-LC (Kan) plasmid.

6.2 Materials and methods

All ligations, SDM procedures, *E.coli* transformations and subsequent plasmid extracts were performed as described in sections 3.6, 3.7, 3.8 and 3.9 respectively.

6.2.1 Extension of clone pB12.1

(i) Generation of PCR amplicons

Subtype B PCR amplicons of varying sizes were generated with primers possessing Xho I RE sites, to further extend clone pB12.1 using sticky-ended cut and ligation techniques. RNA was extracted from a commercial vaccine and reverse transcribed (3.2). Taq PCRs were performed to amplify cDNA (3.3.2) and products were visualised on a 2% agarose gel (3.4). Primers used in the generation of each PCR amplicon, and their positions within the genome are listed in table 10. All PCR amplicons were digested with Xho I (3.10).

Table 10. Sequence and nt positions of primers used to generate each amplicon. Nucleotide position within genome denotes the binding site at the 5' end of the primer.

PCR Amplicon	Primer name	Sequence 5' to 3'	Nt position within genome
1	B12.1 xho+	gttactcagacactcgagatgctatgac	12083
	B12.5 xho -	caggatattctcgagctgtccttgac	12465
2	B12.1 xho+	See Amplicon 1	12083
	B12.8 xho -	gtacacacttgtctcgagtgatgtgc	12769
3	B12.1 xho+	See Amplicon 1	12083
	B13.04 xho -	cgacagctatctcgagtagccttatag	13003
4	B12.1 xho+	See Amplicon 1	12083
	B13.47 xho -	gttttttataactaatgctcgagttttccctattgg	13429
5	B12.5 xho+	gtcaaggacagctcgagaatatcctg	12465
	B13.47 xho -	See Amplicon 4	13429
6	B12.8 xho+	gcacatcactcgagacaagtgtgtac	12769
	B13.47 xho -	See Amplicon 4	13429
7	B13.04 xho+	ctataaggctactcgagatagctgtcg	13003
	B13.47 xho -	See Amplicon 4	13429

(ii) Ligations

A Sal I RE site had been introduced to clone pB12.1 at 12.1kb during a previous study.

The clone was digested with Sal I, allowing the various amplicons with complimentary RE Xho I digested ends to ligate (figure 12). Ligations were carried out in the presence of both restriction enzymes to prevent self ligation (3.6) and products were used to transform *E.coli.* Bacterial growth took place at room temperature. Resulting colonies were screened by Taq PCRs (3.3.2) within the extending sequence. Purified and extracted plasmids were screened across the junction between the original and the extending sequence to verify amplicons had ligated in the correct orientation.

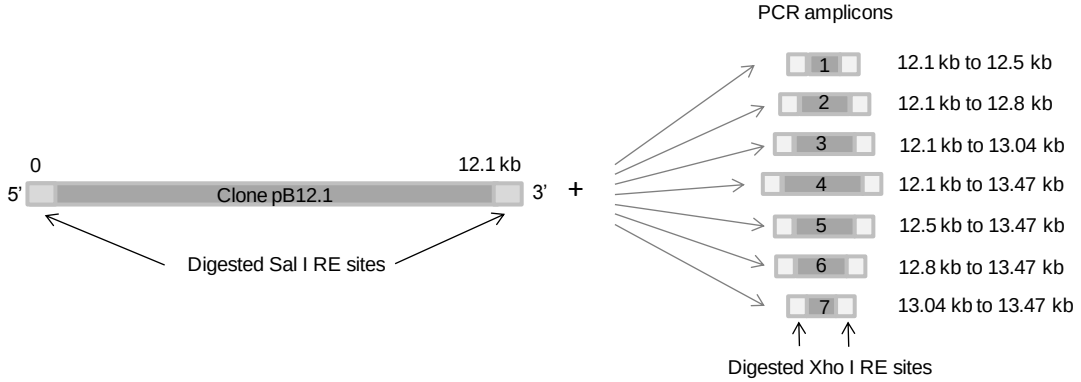

Figure12. Diagram illustrating the various sized PCR amplicons (1-7) used to extend partial clone pB12.1.

6.2.2 Overlap PCR

(i) Generation of PCR amplicons.

Clones pB12.1 and pB12-13 were utilised in the generation of subtype B PCR amplicons, for use in overlap PCRs. Pfu PCRs (3.3.1) were employed to generate blunt ended amplicons which contained a common region of sequence, within the plasmid, from which the overlap PCR would extend (figure 13).

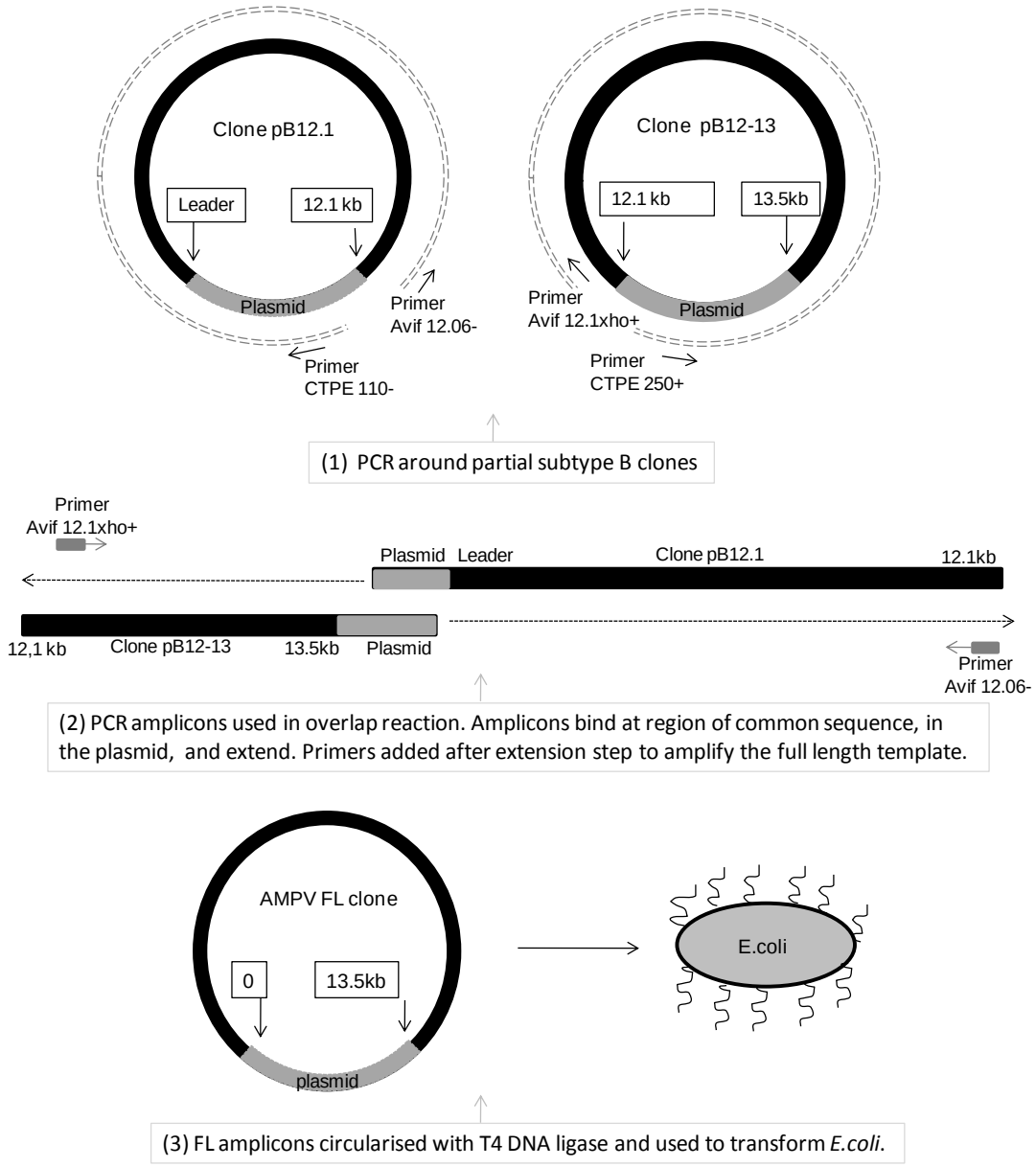

Figure13. Overlap PCR. (1) PCR amplicons generated from partial subtype B clones pB12.1 and pB12-13. (2) In the overlap PCR amplicons bind at common region of sequence in plasmid and extend. Primers are added, after an extension stage, to amplify the FL template. (3) FL amplicons circularised with T4 DNA ligase and used to transform *E.coli*.

The PCR amplicons from clone pB12.1 and clone pB12-13 were generated following the cycle listed in 3.3.1, with a 14 minute and three minute extension, respectively, at step five. Primer sequences are listed in table 11. Products were viewed by UV

following gel electrophoresis. Amplicons were treated with EXO (appendix) and *Dpn1* (3.7 iii) to remove excess primers and original plasmid template.

Table 11. Sequence of primers used in overlap PCRs. Nucleotide position within genome denotes the binding site at the 5' end of the primer.

Primer name	Sequence 5' to 3'	Nt position within genome	Function
Avif 12.06 -	atggttttgaggtactccgtg	12027	Amplification of product from clone pB12.1
CTPE 110 +	cgccgacatcaccgatggggaag	N/A	Amplification of product from clone pB12.1
Avif 12.1 xho +	gttactcagacactcgagatgctatgac	12083	Amplification of product from clone pB12-13
CTPE 250 -	gaacatgctggcgtaatagcgaag	N/A	Amplification of product from clone pB12-13
Avif 12.06 -	atggttttgaggtactccgtg	12027	Amplification after overlap extension
Avif 12.1 xho +	gttactcagacactcgagatgctatgac	12083	Amplification after overlap extension

(ii) Overlap PCR

Overlap PCRs were performed using BIO-X-ACT and Pfu Turbo as described in 3.3.1 and adjusting the extension times for amplification of 13kb. Varying concentrations of PCR amplicon generated from clone pB12.1 were used in reactions with a standard concentration of amplicon generated from clone pB12-13. Primers were added to the reaction after the fifth cycle followed by a further 25 cycles to amplify the FL template (see table 11). Products were visualised on a 0.8% agarose gel under UV (3.4). Potential FL amplicons were circularised using T4 DNA ligase (3.6) before *E.coli* transformations.

6.2.3 Cloning into pSMART-LC (Kan) plasmid

(i) Cloning overview

FL subtype B sequence was amplified in four sections either from clone pB12.1, clone pB12-13 or from a commercial vaccine, and named amplicon A, B, C and D. All primers contained a RE Sal I site, to allow ligation of the amplicon with Xho I digested pSMART1 plasmid. Pfu Turbo PCR's were performed using the cycle described in 3.3.1, with a four minute extension (amplicons A, C and D) and a 2 minute extension (amplicon B) at step 5, and a reduction to 15 cycles at step 7 for

all. Following visualisation on a 1.5% agarose gel (3.4), each product was digested with *Dpn1* if amplified from a partial subtype B clone (3.7iii), digested with RE Sal I (3.10) and finally cleaned with ABgene's Ultra PCR Clean-up kit (cat no. AB-0833) according to the supplier's protocol. Prior to each ligation reaction, plasmid was digested with RE Xho I (3.10). Ligations were performed at 14^{o}C overnight, in the presence of RE Xho I and Sal I to prevent self ligation and products used to transform *E.coli*. Resulting colonies were grown on agar containing Kanamycin antibiotic, and were initially screened in pools. In brief five colonies were picked, using sterile tips, into 25μl of tissue culture water, boiled at 95^{o}c for five minutes and centrifuged at 1000rpm for three minutes. Supernatant (2μl) was then used in GoTaq PCRs (3.3.2). Screening primers were chosen either side of the ligated junction, between the original and the introduced sequence, to isolate constructs containing the insert in the correct orientation. Individual colonies from positive pools were then screened directly using the same PCR enzyme, primers and cycle. Sterile tips were again used to pick a small amount of each colony, which was added directly to the tube containing the PCR mixture. Colonies were grown in liquid culture (3.8 i) and plasmid extracted (3.9). Constructs were digested by RE to ensure no large areas of sequence had been lost during the cloning process (3.10). Following each successful ligation, an Xho I site was re-introduced to the new plasmid, by primer SDM. This enabled the next region of sequence to be added using the same method.

(ii) Plasmid preparation

In preparation for ligations with subtype B AMPV sequence, the linear kanamycin-resistant pSMART plasmid was phosphorylated using T4 polynucleotide Kinase (Promega, cat no M4101) following the supplier's protocol. Plasmid was then circularised with Clonesmart ligase and *E.coli* transformations performed *(3.8).* An

Xho I RE site was introduced to allow ligation with PCR amplicons containing Sal I RE sites. This plasmid was named pSMART 1.

(iii) Cloning of amplicons A-D into pSMART plasmid

All primers used for SDM, screening PCRs and the generation of amplicons A, B, C and D, are listed in table 12.

Amplicon A (8.08 kb -12.083kb)

Amplicon A was generated from a commercial vaccine using primers (B8.08 sal + and Av12.1 sal–) corresponding to the viral genome sequence at position 8080kb to 12,083kb. Following ligation with pSMART 1 and *E.coli* transformations, colonies were screened for successful clones using primers pSMART 1700 + and B8.38-. A correct construct was isolated, purified and named pSMART Ba. An Xho I site was introduced into this plasmid at position 12079 kb by SDM, using primers B12.09 xho + and B12.09 xho - . Presence of the Xho I site was confirmed by restriction digest.

Amplicon B (12.083kb -13.5kb)

Amplicon B was generated from clone pB12-13 and was introduced into pSMART Ba using exactly the same procedures as described for the introduction of amplicon A into pSMART 1. Primers Av12.1 sal + and CTPE 110 sal + were used to generate the amplicon, primers B11.5+ and Av12.2 - were used to screen for positive clones and primers B8.0 pSMART xho + and B8.0 pSMART xho – were used to re-introduce the Xho I site. The plasmid generated in this stage was named pSMART Ba-b.

Amplicon C (4.04kb – 8.78kb)

Amplicon C amplified from clone pB12.1 was introduced into pSMART Ba-b using exactly the same procedures as described above. Primers B4.0 sal + and B8.08 sal

– were used to generate the amplicon, primers B7.36+ and B8.38- were used to screen for positive clones and primers B4050 xho + and B4050 xho – were used to re-introduce the Xho I site.The plasmid generated in this stage was named pSMART Ba-c

Amplicon D (Leader – 3.994kb)

Primers CTPE 240 sal – and B4.0 sal – were used to generate amplicon D from clone pB12.1, from the leader end, including the T7 promoter sequence, to position 3994. This was ligated with construct pSMART Ba-c. Primers FAB 2+ and FAB 4 - were used to screen colonies for the inserted region. Finally, potential FL clones were digested with RE EcoRI to confirm the presence of the complete subtype B sequence. A clone containing a cDNA copy of the FL AMPV subtype B genome was identified and named pSMART Ba-d. The purified plasmid was mapped by PCR and sequenced in its entirety using the subtype B sequencing protocol developed in Chapter 4. SDM was performed using mega primers to correct several nt mutations and to fill small sequence gaps at the junctions between inserted amplicons. A plasmid corresponding to the exact desired AMPV subtype B sequence was named pSMART-AMPV-B.

Table 12. All primers used during the cloning of AMPV-B sequence with pSMART plasmid. Genome position denotes the binding site at the 5' end of the primer.

Primer Name	Sequence 5' to 3'	Genome position
FAB 2+	atgactatgtgttctgtgatactgcagc	3935
B 4.0 sal –	taagtcgacgttgatatgttttggttgc	3994
B 4.0 sal +	catatcaacgtcgacttacccttgcaaag	4004
B4050 xho +	cgacttaccctcgagaagttagcac	4016
B4050 xho -	gtgctaacttctcgagggtaagtcg	4016
FAB 4-	ctcaactgatgtagcccatgttgc	4537
B7.36+	gaaagaagaagaacagcacacaacag	7323
B8.08 sal +	aaactcgttggtcgactcctaaatcg	8080
B8.08 sal –	gatttaggagtcgaccaacgagtttgc	8078
B8.38-	gagcactcttcctgttttctccaacaaac	8367
B11.5+	ctcaaatgcagagagaattgcaactgag	11532
B12.09 xho +	gtgataatgctcgagtactcagacag	12070
B12.09 xho -	ctgtctgagtactcgagcattatcac	12070
Av 12.1 sal +	gttactcagacagtcgacatgctatgac	12083
Av 12.1 sal –	gtcatagcatgtcgactgtctgagtaac	12083
Av 12.2-	gtcgtaggctagtacatgcc	12261
CTPE 110 sal +	cttccccgtcgacgatgtcggcg	N/A
CTPE 240 sal –	attacgccagcgtcgactttcctgcg	N/A
pSMART 1700+	tacggctacactagaagaacagtatttgg	N/A
B8.0 pSMART xho +	gcttgaattcctcgagtcctaaatcg	N/A
B8.0 pSMART xho -	cgatttaggactcgaggaattcaagc	N/A

6.3 Results

6.3.1 Ligations

Products of a good yield were obtained for PCR amplicons 2, 3, 4, 5 and 6. Double bands were seen for PCR reactions 1 and 7, therefore the correct amplicon was isolated and extracted from the gel using Recochip spin columns before further applications.

Very few colonies grew following transformation of *E.coli* with any ligation product and those that did grew slowly. Screening of colonies from ligations with amplicons 2, 3, 4, 5, 6 and 7 by PCR were negative. The ligation with PCR amplicon number 1 generated just one colony. This was positive for the insert by PCR screening however, following extraction of the plasmid from this positive colony, sequencing revealed that the amplicon had been introduced to the plasmid in the wrong orientation.

6.3.2 Overlap PCRs

PCR amplicons were obtained from both partial clones A and B, but the larger amplicon generated from clone pB12.1 appeared slightly weaker. Attempts to improve yield were made by making variations to the PCR cycle and template volume, by using both Pfu Turbo and BIO-X-ACT polymerases, and performing internal nested PCRs, however only slight improvements were achieved. Overlap PCRs only generated weak amplicons with BIO-X-ACT polymerase and with a larger volume (8µl) of PCR template from clone pB12.1. Internal nested PCR's performed with polymerases BIO-X-ACT and Pfu Turbo did not increase the intensity of the overlap amplicons. Transformation of *E.coli* did not result in the growth of any colonies.

6.3.3 Cloning into pSMART plasmid

(i) Plasmid preparation

pSMART 1 was recovered from transformed bacteria, which was confirmed to contain the Xho I site by restriction enzyme analysis.

(ii) Generation of PCR amplicons A-D for ligation into pSMART plasmid

PCR products were successfully generated for ligation with pSMART 1. Amplicons A, C and D were of the approximate predicted size of 4kb, and section B of approximately 1.5 kb.

(iii) Cloning amplicons A-D into pSMART plasmid

E.coli were readily transformed with each of the amplicons yielding many positive colonies. Figure 14 shows results of PCRs performed on colonies obtained after transformation of *E.coli* with amplicon B. Initial screening of pooled colonies resulted in nine positive PCRs from 14 (1,2,3,5,7,8,9,11,and 14, figure 14), each producing a product of the expected size (700bp). The colonies yielding a positive result in pools

1 and 2 were then screened individually. Two colonies were positive from five in pool 1 (3 and 4, figure 15) and one from five in pool 2 (7, figure 15).

For each of the subtype B amplicons highly similar rates of successful cloning were observed (data not shown).

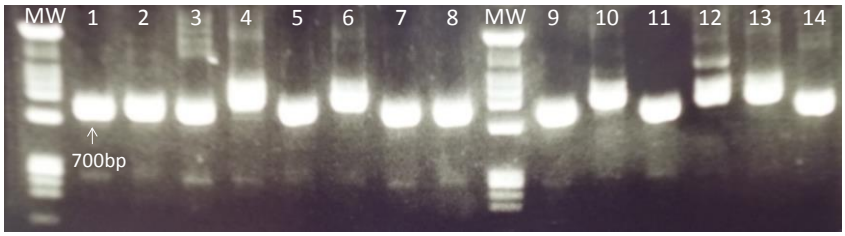

Figure 14. Amplicon B specific PCR screening of pooled bacterial colonies. Each lane (1-14) is representative of pools of five colonies. Positive bands of the expected size (approx 700bp) were obtained in lanes 1, 2, 3, 5, 7, 8, 9, 11 and 14. Molecular weight markers are indicated (MW) (appendix).

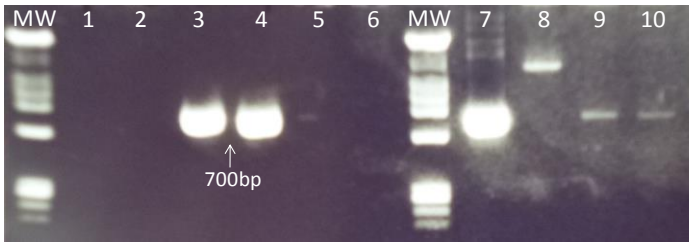

Figure 15. Amplicon B specific PCR screening of individual colonies of pools 1 and 2 figure 14. Positive bands of the expected size (approx 700bp) were obtained in lanes 3, 4 and 7. Molecular weight markers are indicated (MW) (appendix).

(iv) SDM's to introduce Xho I sites to plasmids containing amplicons

SDM's performed to introduce an Xho I site were successful. Figure 16 shows a positive SDM product following the addition of an Xho I site at 12.1kb in pSMART Ba. Figure 17 shows PCR products (700bp) that had been generated across the modified sequence, from colonies transformed with this SDM and Figure 18 shows confirmation of the modified sequence by Xho I digestion of the PCR product (700bp PCR product digested into two parts 500bp and 200bp).

SDMs on all other plasmids containing the subtype B amplicons were equally successful producing highly similar results (data not shown).

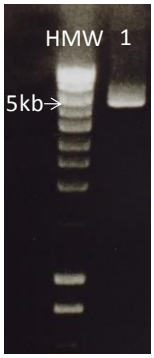

Figure 16. SDM reaction on pSMART Ba to introduce an RE Xho I site at position 12.1kb. Bands of approximately 5kb were obtained as expected. A high molecular weight marker is indicated (HMW)(appendix).

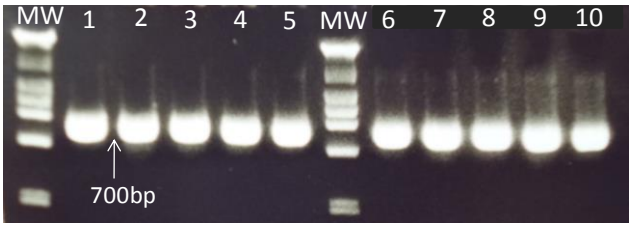

Figure 17. PCR screening of ten colonies (lanes 1-10) transformed with an SDM product to introduce an Xho I site in pSMART Ba. All ten colonies (lanes 1-10) produced the expected size of 700bp. Molecular weight markers are indicated (MW) (appendix).

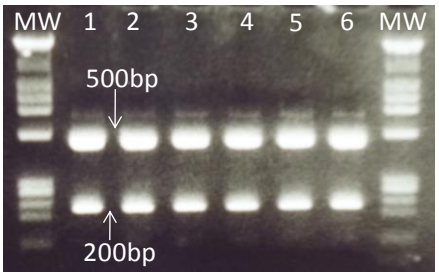

Figure 18. Xho I restriction digestion of PCR products 1-6 figure 17. The introduced sequence was confirmed by the presence of a 500bp and 200bp product. Molecular weight markers are indicated (MW) (appendix).

(v) FL clone

One of two constructs giving a correct RE profile (named pSMART Ba-d, data not shown) was mapped in full by PCRs (figure 19). Products of the predicted size were sequenced. Sequence was almost identical to that of the template sequence used to generate PCR amplicons. SDM successfully corrected small errors and gaps resulting in a FL subtype B cDNA clone of the desired sequence.

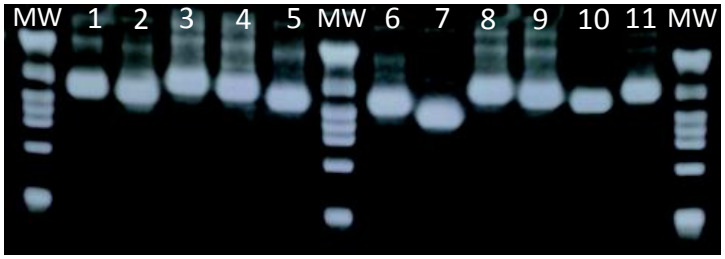

Figure19. PCRs mapping the full length sequence of pSMART Ba-d. Products 1-11 contained overlapping sequence that covered the length of the AMPV- B genome from the leader to the trailer respectively. Molecular weight markers are indicated (MW) (appendix).

6.4 Discussion

Two attempts were made to obtain a FL cDNA clone for AMPV subtype B from partial clones developed during a previous project (82). Firstly digest and ligation techniques were employed to extend clone pB12.1, containing subtype B sequence from the leader end up to 12.1kb. Attempts to extend this any further during the previous project had proved impossible. Here ligations were performed with small amplicons in an attempt to add a little sequence at a time and to determine possible problematic regions in the L gene. *E.coli* transformations with ligated products resulted in poor colony numbers, and very slow growth, similar to that seen in the previous project. Ligation 1, with an amplicon from 12.1kb to 12.5kb, resulted in a single colony which gave a band of the expected size when screened within the inserted amplicon. However PCRs performed on the extracted plasmid across the

junction between the original and the introduced sequence were negative Sequencing confirmed that the insert had ligated with clone pB12.1 in the wrong orientation. A second attempt to gain a FL subtype B cDNA copy was performed using clone pB12.1 and another partial clone, clone pB12-13 in overlap PCRs. Only weak products were generated from overlap reactions. These had been performed with a larger volume of template for clone pB12.1 using polymerase BIO-X-ACT. Further attempts to increase the intensity of the overlap amplicons were made by internal nested PCRs but with no improvement. *E.coli* transformations failed to result in the growth of any colonies.

Whilst these procedures could have been repeated, it seems highly likely that the same result would have been obtained. Much time and effort was devoted to developing a FL cDNA copy in the previous project, but little or no colony growth and sequence deletions due to bacterial instability were continually encountered and became worse with each sequence addition. Further attempts here to gain a FL cDNA copy with the clones developed during that project gave the same outcome with regards to poor or no colony growth. It was decided to focus efforts on cloning with a low-copy kanamycin resistant plasmid (pSMART-LC (kan), Lucigen) specifically designed for the cloning of toxic or large inserts. Cloning with this vector was successful in a study which described the resistance of a single gene segment of the influenza A virus to cloning in a plasmid which had been used successfully for all other gene segments of the virus (244). In that study attempts to clone the segment were first made using different bacterial strains, various culture media, various temperatures, and various antibiotic concentrations with no success. Three different commercial plasmids were then tested and only pSMART-LC (Kan) incorporated the correct length gene segment. Here, amplicons were generated to introduce subtype B sequence in four pieces. These were ligated with the pSMART plasmid in different orders, however ligation of sequence in the order 8kb to12.1kb (amplicon A), 12.1kb to the trailer (amplicon B), 4kb to 8kb (amplicon C) and finally

the leader to 4kb (amplicon D) was the most successful and was first to produce a FL cDNA clone. *E.coli* transformations resulted in healthy colony growth and numbers, and screening PCRs identified several correct constructs for each ligated amplicon. RE digests performed at each stage and after the ligation of the final amplicon verified that no large areas of sequence had been deleted. Sequencing revealed a small number of mutations and gaps which were corrected by SDM. A FL cDNA copy of the desired sequence was then obtained.

This is the first successful generation of a FL cDNA AMPV-B copy and is a major step in the development of an AMPV-B RG system.

Chapter 7

General Discussion

Commercially available live attenuated vaccines, developed following the emergence of AMPV in the 1980's, are used by poultry producers worldwide. Whilst they have been an efficient means of keeping AMPV under control, recent findings suggest updated improved vaccines may be needed (10, 36, 39, 42, 77, 123, 149, 190, 229).

Vaccines have been developed from both turkey and chicken derived isolates and it has been hypothesised that they are more efficient in the species from which they were derived. The first part of this study aimed to determine if host specific motifs existed in the genomic sequence of AMPV's, which then may have confirmed the need for vaccine species matching. A molecular comparison of chicken and turkey derived AMPVs, including field isolates and commercially available vaccines, was performed. To enable the molecular comparison a FL protocol for the amplification and sequencing of subtype B viruses had to first be developed. Here, RT-PCRs were performed on a commercial vaccine, using previously known subtype A and B sequence to aid in the choice of primers. These primers were successful in the amplification and completion of the FL subtype B sequence. Subtype B specific primers were then designed from this sequence for an efficient RT-PCR and sequencing protocol. These PCR's worked efficiently, and along with an already established protocol for subtype A AMPV's, the full genome sequence of a range of subtype A and B field strain isolates and commercially available vaccines of chicken and turkey origin were determined. Unfortunately just one chicken-derived subtype A AMPV was sourced for the study therefore a significant comparison of subtype A chicken and turkey AMPV's could not be performed. That said, sequence obtained

from this single isolate was very similar to that of the turkey isolates. It possessed 34 nt which were not present in the turkey derived isolates. Of these 34 changes 11 were coding (three in the P ORF, three in F, two in L and one in each of the M, SH and G ORF's), two were located in the region before the translation start codon of the N ORF, one between the SH translation stop and G translation start codons, and one between the M2 translation stop and SH translation start codons. The remaining 19 were silent. Further FL sequencing of chicken AMPV isolates would be needed to determine whether these were species specific markers for subtype A chicken AMPV's, or just individual strain differences. Random, individual sequence differences were observed throughout all of the turkey subtype A AMPVs.

Although a sufficient number of subtype B isolates were available for the comparison study, no species specific motifs in the viral sequences were observed.

The lack of chicken derived subtype A isolates may be because it could be less prominent in chickens than subtype B or because isolation may be more difficult. A survey of AMPV's in Western Europe, in both chicken and turkey flocks, found that subtype B was dominant (123), similar to findings from Brazil and Israel (11, 48, 229). Furthermore, studies which compared the pathogenesis of both subtypes in broilers suggested subtype B may be more invasive (8, 77). The authors observed clinical signs in more birds, a wider tissue distribution pattern and longer persistence following challenge with subtype B than with subtype A. dos santos et al (2012) suggest differences exist in virus virulence and replication abilities between the subtypes, which may have played a role (77). As subtype B AMPVs have been reported to generate more clinical signs and persist for longer, isolation may be more successful than that of subtype A. Whilst many studies have successfully isolated AMPV from chickens, in un-complicated infections clinical signs often do not develop and the virus can go unnoticed. SHS can develop later as a result of AMPV infection, but by this time the virus may have cleared (121). Attempts to isolate virus

must be made at the onset of clinical disease, from birds in the flock not yet exhibiting signs (57).

In findings not related to species specificity, the comparison of turkey isolate TA:1 and its vaccine derivative TA:8 revealed eight nt differences, six of which changed the aa codon (one in the M ORF, three in F and two in L). As mentioned, these may have evolved during adaptation to cell culture, or during the attenuation process. A small number of mutations have previously been described following the attenuation of virulent virus in the development of commercial vaccines. The attenuation of subtype A field strain TA:5 entailed only nine nt mutations, of which four were coding, in the development of vaccine TA:6 (36).The generation of a subtype B vaccine involved 18 nt substitutions, of which ten were responsible for aa changes (206). However the latter vaccines, which are still commercially available, have been successfully used in the control of AMPV, whilst vaccine TA:8 failed to offer adequate protection. Previous studies have found that single mutations can be responsible for increased protection or virulence (29, 167). It is possible that one, or a combination of the mutations were responsible for an over attenuation of the virus, leading to an un-protective vaccine. Introducing the mutations, in a step wise fashion by RG, to a FL cDNA clone of field isolate TA:1 may enable the identification of those responsible for over attenuation, and give further insight into the factors involved in attenuation and protection.

It was of interest to find a turkey-derived vaccine (TB:5) and a chicken-derived field isolate (CB:3) with almost identical sequence. This included a mutation causing an early stop codon in the SH ORF, which had been shown to occur in the vaccine but not its progenitor virus in a recent study (206). There were only two definite nt differences in the P and G ORFs at positions 2001 and 6484 respectively, and these were non coding. Several nt ambiguities existed, therefore the isolates may possibly differ at these positions. With this high similarity it seems likely that the

chicken field strain was a derivative of the turkey-derived vaccine. In other observations turkey field isolate TB:2 had an altered transcription start sequence for the SH gene, and was also found to have more individual differences compared to other isolates. This is a relatively recent isolate that has been shown to avoid vaccine induced immunity (39). In that study a subtype B vaccine offered protection to turkeys following challenge with an earlier Italian isolate, TB:1, whilst protection was inadequate following challenge with TB:2. These findings confirm the need for continuing assessment of field and vaccine strains.

In an attempt to determine the sequence of the 3' leader and 5' trailer extremities a method of RNA circularisation was used. Sequence was tentatively determined for a subtype A isolate. This was compared to previously reported leader and trailer sequence of a subtype A AMPV isolate and found to have one nt difference in the leader extremity and two possible differences in the trailer extremity. However this method alone was not efficient for the precise determination of the extremities and many problems including failure of PCRs, poor sequence reads and missing bases were experienced despite alteration of various conditions. Therefore the extreme leader and trailer extremity sequence was not taken into account in the comparison studies. It may be possible that during the ligation the complimentary ends of the genome ligated together, thereby hindering the ability to obtain clear precise sequence of the extreme leader and trailer ends. Cloning the PCR products from the ligated junction may have led to clearer sequence data, however anomalies in the sequence of the genomic termini of hMPV following cloning were reported in a study by Herfst et al (2004) (114). In addition the circularisation technique alone does not permit the determination of the exact terminal residue of the leader nor the trailer, therefore alternative techniques such as 5' RACE also need to be performed. A recent study reported a simple one-step method for the determination of both the

leader and trailer ends of single-stranded negative-sense RNA viruses, using 3' base tailing of both the genome and its positive replication intermediate (27).

Passage of turkey AMPV's in chicken TOC was performed to investigate the potential of turkey AMPV's to acquire chicken specific AMPV sequence motifs. However, the comparison study performed in parallel showed that no species specific motifs existed therefore, no information could be drawn from the passage experiments relating to this issue. However, it was interesting to note that TOC passage produced more mutations in vaccine strains than in field strains. Therefore the vaccine strains, which had been pressured to grown in a foreign cell line in the attenuation process, appeared to be more unstable once back in a host respiratory cell line. It would be interesting to investigate the effects of these mutations *in vivo* with respect to gain or loss of virulence

The development of a FL RT-PCR and sequencing protocol enabled the fast determination of the viral genome sequence for subtype B AMPV's. The data gathered will be of use in FL sequence comparisons with future field strains, helping monitor virus evolution. A larger number of FL sequences of both subtype A and B isolates from both species, including a larger number of recent isolates, would allow a more complete comparison study to be undertaken. However it may be reasonable to conclude here that species specific motifs do not exist between chicken and turkey-derived AMPV isolates. None were seen in the subtype B isolates, and given the close relationship between subtype A and B AMPV's it is likely the same would have been true for subtype A if more chicken isolates had been available for the study. There remains the theoretical possibility that certain combinations of apparently unrelated mutations might cause some species specificity, but nonetheless the consideration of factors such as the level of attenuation and the virus subtype are likely to be of greater importance in the

development of a vaccine than the species from which the AMPV was derived. Furthermore, the two species may have differences in their local cell-mediated immune reactions and systematic T cell activity, which may enable chickens to clear virus and recover faster than turkeys (189). The authors have suggested vaccines which specifically stimulate local cytotoxic CD8 + T cells may be more efficient for chickens than those which stimulate humoral immunity.

The second part of this study focused on the virulent field strain, #8544, from which the two major commercially available live subtype A vaccines have been developed. To enable preliminary studies into an improved modified vaccine closely related to this field strain, a FL #8544 DNA copy was developed by SDM with a FL copy of one of its vaccine derivatives (Vaccine 1). However, it could not be recovered in the Vero cell based RG system, unlike the FL DNA copy of Vaccine 1, thereby hindering further investigations. It was known that nine mutations had arisen during the development of the vaccine from strain #8544, some which would have been responsible for a reduction of virulence and some of which would have arisen during the adaptation to the cell culture. To determine which of the mutations had been permissive for growth in Vero cells, the vaccine specific sequence substitutions were re-introduced by SDM to the FL #8544 DNA copy. It was found that a FL #8544 derivative with a single non-conservative aa substitution in the M2 second ORF, at genome position 5140, could be recovered by RG and grew readily in Vero cells. To determine if the mutation had rendered M2:2 non-functional, or had simply changed it to one more suitable for growth in Vero cells, the FL #8544 DNA copy was modified to corrupt either the first AUG start codon only, or both the first and second AUG start codons of M2:2, to prevent expression. Both of these FL derivatives were recovered in the RG system and readily yielded virus. These findings suggest that for an #8544 FL the presence of M2 second ORF expression prevents virus rescue in Vero cells.

It initially appears counterintuitive that the loss of M2:2 should facilitate virus replication because a previous study showed that M2:2 deletion in AMPV subtype A led to reduced replication (146). However for AMPV subtype C, deletion of M2:2 from a Vero adapted virus led to largely normal replication in Vero cells, but again much inhibited replication in turkeys (242). Similarly a study of the related respiratory syncytial virus (RSV) showed that loss of M2:2 resulted in markedly inhibited growth in the tracheas of mice and cotton rats as well as Hela, HEp-2 and MRC 5 cells, whereas in Vero cells virus replicated efficiently and produced larger than typical plaque formations. The authors suggested this cell type restriction in an M2:2 deletion mutant may have been because the suggested normal role of M2:2 in assisting switching to virus replication, was substituted by Vero cell “host factors” not present in the other cells and tissues (116). Interestingly in a previous AMPV study of Vaccine 1, ability to replicate in avian TOC was conclusively shown to have been lost (165) and it was assumed that replication and ensuing immune priming was occurring in another unspecified location. In the light of the RSV findings, it appears possible that the loss of M2:2 function due to the 5140 mutation may have been responsible for this cell tropism shift in Vaccine 1.

To enable studies into a modified #8544 vaccine using the Liverpool based RG system, it was essential that the virus replicated in Vero cell culture. Replication in Vero cells has played a major role in live AMPV vaccine developments ever since the virus type was first discovered (32). Initially, the cells were used because it became known that Vero passaged AMPVs tended to be attenuated while still inducing protection (33). Studies at Liverpool have shown that AMPV grows to the highest available titres in these cells compared with other primary and continuous cells lines. This finding has more recently been confirmed by others (71). Virus titre is of great importance in vaccine developments because if sufficient titre per cell

culture area cannot be achieved, an otherwise promising vaccine candidate will be rejected on technical and cost grounds.

There are contrasting views about the ability to use Vero cells for isolation of subtype A and B field AMPVs. At Liverpool, direct isolation has proved impossible even when using a range of available Vero cell line sources. However some groups have had more success, notably if inoculated Vero cells are serially trypsinised and split (9, 136). The reason for the increased isolation success is not clear but, as the presence of trypsin is the main differentiating feature, it may be that the effect of trypsin on either viral surface proteins or Vero cell proteins in the presence of virus, is influential. However given the excessive number of trypsin assisted passages required, it may be that all such isolations are in reality adaptations involving M2:2 mutation. It is interesting that only the Vero adapted viruses of seven sequenced in this study contained M2:2 aa changes likely to affect the protein function. This is suggestive of the notion that Vero cell adaption of AMPVs require such mutations but clearly many more Vero adapted strains would need to be assembled or prepared, then sequenced to be able to justify such an assertion.

The study adds to the speculation as to which of the coding mutations, identified by sequencing (36), in the conversion of #8544 to vaccine 1 (234) were responsible for the observed attenuation. Coding mutations were limited to the F and M2 genes and, in light of above discussions, it is possible that the 5140 mutation alone attenuated due to the tropism change already described (165). Vaccine 1 did not replicate in avian trachea and it was deduced to be replicating, and presented to the immune system, in unspecified tissues. This might lead to the future prospect of rapidly producing AMPV vaccines from current field strains simply by M2:2 modification. This could be conveniently achieved by making a FL copy by modification of an existing FL, adding the M2:2 change, then recovering virus in the

RG system . This could be especially important in the context of the growing evidence of field strain evolution allowing avoidance of immunity from older vaccines (39). However the role of the fusion protein also needs consideration. In another study focussing on induced protection in vaccine candidates, the substitution of the #8544 F gene into a German isolated AMPV led to a chimera showing increased protection but also a small virulence increase (168). While that chimera virus was much less virulent than #8544, it would probably have been greater, had G expression not been reduced due to SH gene end mutation. The role of F in the virulence of Vaccine 1 and AMPVs in general has not yet been fully investigated and this is likely to be a focus for the group's future studies and vaccine developments. The foremost practical outcome of the study is that AMPV field virus # 8544 can be adapted to Vero cells by alteration of the M2:2 second open reading frame. This is a major step forward because the two major subtype A AMPV live vaccines available worldwide are empirical adaptations of the #8544 field strain, hence this finding will open the way to specific rational modification of #8544 for vaccine development purposes by Vero cell based RG. It might further offer a mechanism for Vero adaption and attenuation of non #8544 field strains.

The final chapter of this study aimed to continue with previous developments towards a RG system for AMPV subtype B, to enable studies such as those described above using the subtype A RG system. An increasing number of detections in the field and reports of vaccine failures highlight the need for a similar system for AMPV-B (10, 36, 39, 42, 77, 123, 149, 190). Clones with partial subtype B sequence were generated during a previous study at Liverpool. These were employed here in continued attempts to obtain a FL cDNA copy. One attempt employed standard digest and ligation techniques to extend a clone already containing 85% of the subtype B genome up to 12.1kb. This had been attempted in the previous project but sequence in the trailer end of the L gene had appeared to

be toxic to bacterial cells resulting in little or very poor growth in *E.coli*. Smaller amplicons were generated in the present study to try to gradually add small pieces and determine possible areas of sequence responsible for bacterial instability. However the same problem of poor colony growth was observed and only one ligation resulted in a possible sequence addition from 12.1kb to 12.5kb. Following PCRs and sequence analysis it was found to be ligated in the wrong orientation. In a second strategy, PCR amplicons were amplified from two partial subtype B clones, which together contained the full genome sequence. These amplicons which had a common region of sequence within the plasmid were used in overlap PCRs. Whilst weak products were observed, no colonies grew following *E.coli* transformations. Various factors have been implicated in the failure to clone certain areas of viral sequence including long repeats, AT-rich sequences or secondary/tertiary structures, promoters or toxic proteins generated by viral sequences (244). The latter factor was suggested to be a possible problem during the previous study from which the clones used here were developed. Certain areas of viral sequence can encode bacterial toxic peptides and therefore cannot be tolerated by E.coli (82). It was decided here to clone sequence with pSMART-LC (Kan) plasmid from Lucigen. This low-copy plasmid (15~20 copies/cell) contains transcriptional terminators at various sites to prevent transcription into or out of the inserted DNA. The successful cloning of difficult regions of sequence with this plasmid had been previously described (244). Cloning with pSMART was fast and efficient with many healthy colonies obtained following transformation of *E.coli* and several correct constructs identified following the ligation of each amplicon. Inserts were ligated with pSMART in different orders however the order 8kb to 12.1kb (amplicon A), 12.1kb to the trailer (amplicon B), 4kb to 8kb (amplicon C) and finally the leader to 4kb (amplicon D) proved to be the fastest and successfully generated a FL cDNA genome copy. This is a major piece of the RG system for AMPV subtype B currently being developed at Liverpool. Successful rescue of subtype B virus will open the door to

studies on subtype B AMPVs and allow modifications of the genome for future recombinant vaccines.

Future work

It would be of interest to determine the FL genome sequence from subtype A AMPVs of chicken origin, should they become available, to enable the completion of the molecular comparison study undertaken in Chapter 4. Furthermore, a simple method outlined in a recent study could be employed to identify sequence of the 3' and 5'genome extremities (27). The determination of FL genome sequences from future AMPV isolates may aid the monitoring of field virus evolution. The subtype B RT-PCR and sequencing system developed here can be employed, along with the already established system for AMPV subtype A . However, Next Generation Sequencing (NGS) may also offer a fast and possibly cheaper method for FL sequence determination of AMPVs and merits investigation. It could also be of interest to investigate whether modification of the M2:2 ORF, performed by RG in Chapter 5, enables the adaptation of current AMPV isolates to growth in Vero cell culture. Furthermore, *in vivo* studies could be performed to determine if M2:2 modification alone sufficiently attenuates AMPV strains and offers adequate protection against virulent challenge. This may lead to the prospect of rapidly producing AMPV vaccines from currently circulating field strains. If the current isolates could indeed be adapted using RG, then it supports the notion that AMPV adaptation to growth in Vero cells, by multiple passage, involves M2:2 mutations. For a study of this kind a wide range of isolates could be sequenced before and after their adaptation to Vero cells, then aligned and compared.

Final components of the subtype B AMPV RG system will be developed in future work followed by attempts to rescue live virus. This tool will be invaluable for elucidating sequences or sequence domains involved in protective immune

responses, pathogenicity or attenuation with an ultimate goal of developing vaccines with improved stability and / or efficacy.

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Appendix

Agarose gel electrophoresis 1.5%

Agarose powder and TBE buffer was adjusted accordingly to achieve a desired %.

- 0.5g Agarose powder[35]
- 35ml 1x TBE buffer[36]

Swirl to mix in a conical flask then heat for 40 seconds in a microwave (full power). Swirl to cool under cold running water, add red safe nucleic acid staining solution[37], swirl to mix and pour into gel tank. Allow gel to set then overlay with TBE buffer.

dNTP solution

- 20µl dATP (100mM)
- 20µl dCTP (100mM)
- 20µl dGTP (100mM)
- 20µl dTTP (100mM)

120µl double processed tissue culture water[38]

DNA load buffer (Ficoll)

- 0.25% Bromophenol blue[39]
- 30% Ficoll type 400[40]

In a total volume of 10ml distilled water

DNA load buffer (Glycerol)

- 0.5% Bromophenol blue[39]
- 30% Glycerol[41]
- 30% 10x TBE Buffer[36]
- In a total volume of 10 ml distilled water

[35] Promega	Cat no. v-3121	Agarose powder
[36] Invitrogen	Cat no. 15581-044	Ultrapure TBE buffer, 10X
[37] Intron Biotechnology	Cat. no. 21141	Red Safe Nucleic Acid staining solution
[38] Sigma	Cat. no. W3500	Double processed tissue culture water
[39] Sigma	Cat no. B-0126	Bromophenol blue
[40] Sigma	Cat no. F4375	Ficoll type 400
[41] Sigma	Cat no0 G5516	Glycerol

EXO / SAP

- 15µl PCR amplicon
- 1µl SAP[42]
- 0.15µl EXO I[43]

Cycle 37°C for 30 minutes, 80°C for 10 minutes, hold at 4°C

EXO

- 0.5µl EXO I[43] / 50µl PCR amplicon

Cycle 37°C for 30 minutes, 80°C for 10 minutes, hold at 4°C

Molecular weight standards (MW) (2µl loaded to gel per run)

- 50µl λDNA/*Hin*d III [44]
- 25µl ΦX174 RF DNA/*Hae* III[45]
- 25µl DNA load buffer (Ficoll) (see DNA load buffer above)

High molecular weight standards (HMW) (2µl loaded to gel per run)

- 50µl λDNA/*Hin*d III[45]
- 30µl Double processed tissue culture water[46]
- 20µl DNA load buffer (Glycerol) (see DNA load buffer above)

[42] Usb	Cat. no. 70073Z	Shrimp Alkaline Phosphatase
[43] Usb	Cat. no. 70092Z	Exonuclease I
[44] Invitrogen	Cat.no 15612-013	λDNA/*Hin*d III
[45] Invitrogen	Cat no. 15611-015	ΦX174 RF DNA/*Hae* III
[46] Sigma	Cat. no. W3500	Double processed tissue culture water

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Avian metapneumovirus M2:2 protein inhibits replication in Vero cells: Modification facilitates live vaccine development

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ABSTRACT

Throughout the world, avian metapneumovirus (AMPV) infection of subtype A is principally controlled by two live vaccines both derived from UK field strain #8544. Improvements of those vaccines by use of reverse genetics technology was found to be hampered by the inability of #8544 to replicate in the commonly exploited Vero cell based reverse genetics system. A systematic reverse genetics based genome modification of a DNA copy of #8544, employing sequence data from a Vero grown, #8544 derived, live vaccine; was used to determine mutations required to facilitate virus recovery and replication in Vero cells. This identified a single coding substitution in the M2:2 reading frame as responsible. Furthermore, ablation of M2:2 was found to elicit the same outcome. M2:2 sequence analysis of seven AMPVs found Vero cell adaption to be associated with non similar amino acid changes in M2:2. The study shows that M2:2 modification of field virus #8544 will enable research leading to improved vaccines. This may have more general application to other AMPV field strains.

1. Introduction

Avian metapneumovirus is a single stranded, negative sense virus of the family Paramyxoviridae which occurs in 4 known subtypes (A, B, C and D). Subtypes A and B cause major disease and economic losses in unprotected poultry throughout most of the world [1–3]. While disease has been largely controlled following the development of live vaccines, it still occurs. Some has been shown to be due to vaccine instability in the field [4] while more recently, virus change or evolution, allowing avoidance of immunity induced by older vaccines, had been demonstrated to be another important factor [5,6]. It is likely that more complete disease control will only come about if more field-stable vaccines can be developed which are also relevant to the prevailing circulating field strains.

The live attenuated AMPV vaccines used to date have derived from virulent field strains which have been adapted to cell culture followed by further virulence-reducing passages [7]. In the process, mutations arise of which it is likely that some are responsible for virulence reduction and others for adaptation to the selected cell culture. More recent vaccine developments have started to make use of AMPV reverse genetics (RG) [8,9] which allows potentially attenuating mutations to be added to a DNA copy of a field virus in a stepwise fashion, by making use of data acquired during previous empirical vaccine developments. An obvious prerequisite of this approach is that field virus is able to replicate in the cell culture of the given RG system.

Replication in Vero cells has played a major role in live AMPV vaccine developments ever since the virus type was first discovered [10]. Initially the cells were used because it had became known that Vero passaged AMPVs tended to be attenuated while still inducing protection [11]. Studies in our group, often in association with commercial vaccine companies, have shown that AMPV grows to the highest available titres in these cells compared other primary and continuous cells lines. This finding has more recently been confirmed by others in the scientific literature [12]. Virus titre is of great importance in vaccine developments because if sufficient titre per cell culture area cannot be achieved, an otherwise promising vaccine candidate will be rejected on technical and cost grounds. Another so far unreported feature of Vero cell culture has been the remarkable genetic stability of AMPVs when serially passaged in those cultures. In our laboratory, AMPVs, some with 1–2 kb insertions, are routinely passaged 5–10 times without any detectable mutations to full genome consensus sequences (currently unpublished).

Previously in our laboratory, one of the two available major commercial subtype A live vaccines was produced from virulent AMPV field virus UK #8544 by adaptation to, and subsequent passage in, Vero cells [7,13,14]. In preparing various candidate vaccines for that study, #8544 had been shown to readily grow in avian tracheal tissue and avian fibroblasts but not Vero cells [7]. Vero adaptation was eventually achieved via a lengthy adaptation to chick embryo

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Table 1
Sequence differences between field strain #8544, #8544 derived Vaccine 1 and derivative mutants.

Position[a]	Region[b]	#8544		Vaccine 1		$\#8544_{vac\ 4.87\text{-}6.37}$		$\#8544_{vac\ 5055}$		$\#8544_{vac\ 5140}$		$\#8544_{vac\ 5929}$		$\#8544_{vac\ 6358}$		$\#8544_{no\ M2:2\ AUG\ 1}$		$\#8544_{no\ M2:2\ AUG\ 1+2}$	
		RNA[c] 1	aa[d]	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa	RNA	aa
2941	F_{TS}[e]	U	none	A	none	U	none	U	none	U	none	U	none	U	none	U	none	U	none
3553	F	U	V	C	A	U	V	U	V	U	V	U	V	U	V	U	V	U	V
3825	F	G	E	A	K	G	E	G	E	G	E	G	E	G	E	G	E	G	E
5055	M2:1	A	K	G	R	G	R	G	R	A	K	A	K	A	K	A	K	A	K
5104	M2:1	U	N	U	N	U	N	U	N	U	N	U	N	U	N	C	N	C	N
5104	M2:2	U	SC1[f]	U	SC1	U	SC1	U	SC1	U	SC1	U	SC1	U	SC1	C	noSC[h]	C	noSC[h]
5140	M2:1	U	N	C	N	C	N	U	N	C	N	U	N	U	N	U	N	U	N
5140	M2:2	U	I	C	T	C	T	U	I	C	T	U	I	U	I	U	I	U	I
5169	M2:1	A	none	A	none	A	none	A	none	A	none	A	none	A	none	A	none	U	none
5169	M2:2	A	SC2[g]	A	SC 2	A	SC 2	A	SC 2	A	SC 2	A	SC 2	A	SC 2	A	SC 2	U	noSC[h]
5929	SH-G	A	none	G	none	G	none	A	none	A	none	G	none	A	none	A	none	A	none
6358	G	U	F	C	F	C	F	U	F	U	F	U	F	C	F	U	F	U	F
10022	L	U	V	G	V	U	V	U	V	U	V	U	V	U	V	U	V	U	V
11624	L	U	Y	C	Y	U	Y	U	Y	U	Y	U	Y	U	Y	U	Y	U	Y

[a] Nucleotide position (antigenome 5′ to 3′).
[b] Region within genome.
[c] Nucleotide identity at genome position.
[d] Predicted amino acid coded.
[e] Transcription start.
[f] M2:2 1st start codon.
[g] M2:2 2nd start codon.
[h] M2:2 1st and 2nd start codons corrupted.
[i] Virus grew in TOC but not Vero cells.

liver cells which itself involved multiple centrifugations and trypsin treatments. After 17 Vero cell passages, the virus produced no disease in one-day-old turkeys yet conferred full protection three weeks later [7] and this, now commercial preparation, is designated Vaccine 1 in this study. Both vaccine 1 and #8544 progenitor were later fully sequenced whereon it was found that nine mutations, had been introduced [15]. This stimulated questions, mainly still unanswered, as to the role of these mutations and furthermore encouraged the notion that investigation of the individual mutations might lead to improved vaccines. With the advent of AMPV reverse genetics, this became a feasibility [8]. It was decided to build on previous empirical vaccine 1 developments [7,9] and make and investigate properties of further candidate vaccines more closely related to the original #8544 field strain.

The current study describes how a full length (FL) copy of field virus #8544 sequence was constructed but then shown not to lead to virus recovery in our Vero cell based RG system. However it was known that #8544 derived vaccine 1 grew readily in Vero cells [7,16] and it was also known from a previous study that a FL DNA copy of that vaccine, and its derivatives, could be recovered by reverse genetics [16]. It was hypothesised that during the conversion of #8544 to Vaccine 1, a single or combination of growth permitting mutations had been introduced. Sequencing showed that nine nucleotide substitutions arose during the generation of vaccine 1, shown in white text in Table 1. Four substitutions altered coded amino acids (genome positions 3553, 3825, 5055, 5140), three maintained the original amino acid (genome position 6358, 10,022, 11,624) and two occurred in non coding regions (2941, 5929) [15]. In addition it was known that virus with the #8544 F gene (covering positions 2941, 3553, 3825) within a German A type AMPV (LAH A) could be recovered in the RG system [9] thereby suggesting that #8544 F sequence was not preventing Vero cell replication. Following a series of logical mutations to a full length copy of field strain #8544, the described study was able to show that a non conservative amino acid substitution in the M2 second reading frame was able to facilitate replication in Vero cell culture. Computer software predicted that glycosylation changes were unlikely to be involved. The negation of M2:2 translation by start codon corruption led to an identical outcome. Sequencing of other AMPV subtype A M2:2 genes suggest possible broader application. These findings open the possibility of new, current-field-strain-related vaccines by reverse genetics that furthermore possess the industrial production advantages afforded by Vero cell culture.

2. Materials and methods

2.1. Full length genome copies

Cloned FL genome copies were all prepared from a FL copy of vaccine 1 (pVc) using region or nucleotide directed mutagenesis as previously described [16]. Region directed mutagenesis made use of high fidelity blunt end PCR amplicons to introduce modified sequence to an already cloned FL copy. This is illustrated in Fig. 1 which depicts the conversion of the Vaccine 1 FL to an #8544 FL. In a related manner, nucleotide directed mutagenesis made use of complementary oligonucleotide pairs to substitute single nucleotides. The details of differences to sequences of FLs are given in Table 1 and details of oligonucleotides are provided in Table 2. The antigenomic oligonucleotide was used in all cases to prime reverse transcription, prior to PCR. Each stage produced parallel clones which were screened for copying errors. Final clones used for attempted virus recovery by RG were sequenced in their entirety [8].

2.1.1. p#8544

An exact cloned DNA copy of virus #8544 was prepared from a FL DNA copy of vaccine 1 (pVc), as outlined in Fig. 1. Briefly two high fidelity RT-PCR copies of #8544 were used in two stages to change the sequence of pVc to that of #8544 between genome positions 2949 and 6416 (using primers M8+ and G6-A ext) and 9945 and

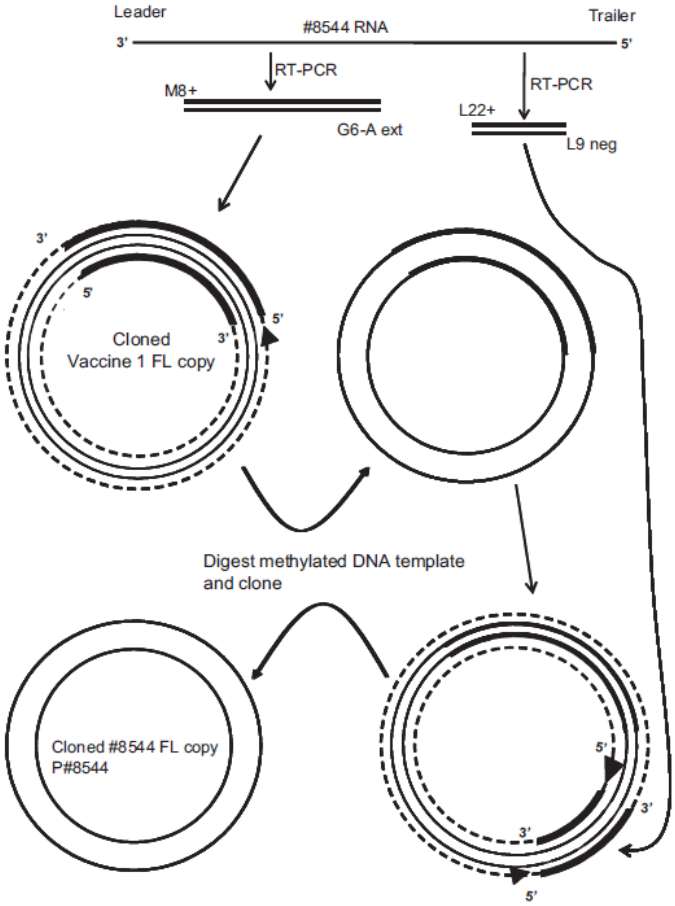

Fig. 1. Schematic representation of the method used to construct a cloned DNA copy of field virus #8544. Vaccine 1 clone was modified by region directed mutagenesis in two stages by two high fidelity RT-PCR generated DNA amplicons, using RNA extracted from virus #8544 as template.

11,736 (using primers L22+ and L9neg). The resultant plasmid was named p#8544.

2.1.2. *p#8544* $_{vac\ 4.87–6.37}$

Vaccine 1 sequence was re-established in p#8544 at positions 5055, 5140, 5929 and 6358 using a high fidelity RT-PCR covering bases 4874–6371 (using primers M2 mid for and G2-A ext) and the resulting plasmid was named p#8544 $_{vac\ 4.87–6.37}$.

2.1.3. *p#8544* $_{vac\ 5055}$, *p#8544* $_{vac\ 5140}$, *p#8544* $_{vac\ 5929}$ *and p#8544* $_{vac\ 6358}$

Four full length copies were generated from p#8544 by introducing Vaccine 1 sequence at positions 5055, 5140, 5929 or 6358 using nucleotide directed mutagenesis employing primers Vac 5055+, Vac 5055 neg, Vac 5140+ Vac 5140 neg, Vac 5929+, Vac 5929 neg, Vac 6358+ and Vac 6358 neg. FL copies were named p#8544 $_{vac\ 5055}$, p#8544 $_{vac\ 5140}$, p#8544 $_{vac\ 5929}$ and p#8544 $_{vac\ 6358}$, respectively.

2.1.4. *p#8544* $_{no\ M2:2\ AUG\ 1}$ *and p#8544* $_{no\ M2:2\ AUG\ 1+2}$

Lastly p#8544 was modified to ablate the M2:2 open reading frame start codon (genome position 5104–5106, AUG converted to ACG), either alone (named p#8544 $_{no\ M2:2\ AUG\ 1}$) or in combination with a further potential downstream start codon (5169–5171, AUG converted to UUG) within the same frame (named p#8544 $_{no\ M2:2\ AUG\ 1+2}$), using nucleotide directed mutagenesis with primer pairs M2:2 5104+ and M2:2 5104 neg or M2:2 5169+ and M2:2 5169 neg. The amino acid sequence of the M2 first open reading frame remained unaltered.

2.2. Virus recovery from FL plasmids by reverse genetics

FL DNA copies were transfected with other components of the AMPV RG system as previously described [8]. In all cases 160 ng of the cloned full length copy was transfected together with the other RG system components. If virus was not recovered, the rescue attempt was repeated both identically and with variations of

Table 2
Sequences of oligonucleotide primers used for reverse transcription, PCR and nucleotide site directed mutagenesis.

Primer name	Sequence 5′ to 3′	Sense	Position of leader proximal terminus (antigenome)
M8+	gaagctgcaataagtgggaag	Antigenomic	2649
M2 mid for	ccagagattcaatgcttgaagaccc	Antigenomic	4923
Vac 5055 +	gagaaaaactcaaaaggcttgccaagatcatac	Antigenomic	5040
Vac 5055 neg	gtatgatcttggcaagccttttgagtttttctc	Genomic	5040
M2:2 5104+	tgacaacgccagtggtaataccc	Antigenomic	5098
M2:2 5104 neg	gggtattaccactggcgttgtca	Genomic	5098
Vac 5140 +	gaagggtgacagcaacaattaagtgcaatgcac	Antigenomic	5125
Vac 5140 neg	gtgcattgcacttaattgttgctgtcaccctc	Genomic	5125
M2:2 5169+	caatgcacttggattgtgtttggttcg	Antigenomic	5151
M2:2 5169 neg	cgaaccaaacacaatccaagtgcattg	Genomic	5151
Vac 5929 +	gacaacactagtgccaaatgataggcaacag	Antigenomic	5910
Vac 5929 neg	ctgttgcctatcatttggcactagtgttgtc	Genomic	5910
Vac 6358+	ctgtactgggtggtttcgattgtatgggcttattgg	Antigenomic	6342
Vac 6358 neg	ccaataagcccatacaatcgaaaccacccagtacag	Genomic	6342
G2-A ext	gcccatacaatcaaaaccacccag	Genomic	6347
G6-A ext	gattatctcgctgacaaattggtcctg	Genomic	6390
L22+	ggttagcagatgagagcagc	Antigenomic	9943
L9 neg	caccttgctcatcatccgccaa	Genomic	11,714

the FL plasmid concentration. To ensure that the system was functioning efficiently, in all cases a standardised control transfection was performed using an SH deletion virus which produces a very clear giant syncytial formation using low power microscopy [17]. Because any cytopathic effect (CPE) is generally obscured by the effects of the transfection agent (Lipofectamine 2000, Invitrogen), all the transfected cells were freeze thawed after 7 days and used to inoculate further Vero cell monolayers.

2.3. Detection of recovered virus

With the exception of the positive control virus, the presence of CPE was investigated from the first Vero cell passage onward. If virus could not be detected by CPE, two further Vero cell passages were made. If virus remained undetectable by CPE in the third Vero passage, evidence of virus recovery was attempted using an established RT-PCR routinely used for sensitive detection of AMPV in diagnostic field samples [18].

2.4. Sequencing of subtype A AMPV M2 genes

To compare M2:2 of different viruses, freezer stored subtype A viruses of known passage history were sequenced. Some had been isolated and passaged in chick embryo tracheal organ cultures, sometimes in combination with passage in other chicken primary cells but none had ever been adapted to Vero cells. These comprised chicken isolate CP1 [19], turkey isolate UK3B [20], the other major subtype A live vaccine also #8544 derived, and Turkadin, a now obsolete vaccine prepared from a UK3B [21]. The other group contained viruses which had been Vero cells adapted and included vaccine 1 and LAH A isolated in Germany [8]. The M2 genes were sequenced using previously described methods [22] and predicted amino acid sequence of the M2:2 reading frames were derived.

2.5. Prediction of M2:2 glycosylation

NetOglyc 3.1 analysis [23] was performed on predicted M2:2 sequences to investigate potential for O linked glycosylation of S and T amino acids.

3. Results

3.1. Evidence of virus after transfecion of FL DNA copies

For p#8544, CPE was not observed after careful observation of transfected cells or in the subsequent two passages. RT-PCR in the G gene also failed to detect evidence of viral genome. The same result was found after repeat transfection, and variations to the concentrations of components of the RG system did not alter that outcome. However for p#8544 $_{\text{vac 4.87–6.37}}$ CPE similar to that caused by vaccine 1 was readily found after one passage of the RG transfection. With p#8544 $_{\text{vac 5055}}$ p#8544 $_{\text{vac 5929}}$ and p#8544 $_{\text{vac 6358}}$ CPE was not observed after careful observation of transfected cells or in the subsequent two passages. RT-PCR in the G gene also failed to detect evidence of viral genome. The same result was also seen after variations were made to the concentrations of components of the RG system. For p#8544 $_{\text{vac 5140}}$, CPE similar to that caused by vaccine 1 was readily found after one passage of the RG transfection. For p#8544 $_{\text{no M2:2 AUG 1}}$ and p#8544 $_{\text{no M2:2 AUG 1+2}}$, CPE similar to that caused by vaccine 1 was readily found after one passage of the RG transfection.

3.2. Sequences of FL used in RG system

All cloned FLs used in virus rescue attempts were sequenced in their entirety and found to be as designed.

3.3. Sequencing of subtype A AMPV M2 genes

The predicted amino acid sequences for M2:2 are shown in Fig. 2. For non Vero grown viruses, the sequence of the M2:2 gene only varied with respect to a conservative R K variation at amino acid residue 25. However both of the two viruses grown in Vero cells had predicted non-similar amino acid substitutions. At residue 12, the hydrophobic isoleucine (I) of #8544 had been substituted with polar threonine (T) in Vaccine 1 and in LAH A, the consensus aromatic amino acid of phenylalanine (F) had become substituted by aliphatic leucine (L).

3.4. Prediction of M2:2 glycosylation

NetOglyc 3.1 analysis showed that none of the 4 S or 2 T residues were likely to be glycosylated. This also was also true for the additional T residue present at residue 12 in Vaccine 1.

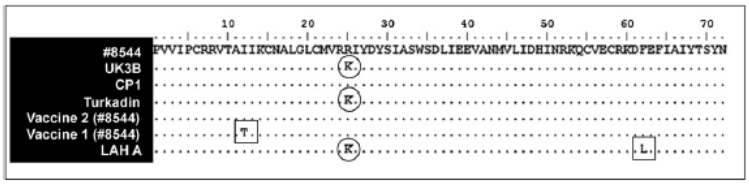

Fig. 2. M2:2 sequences of 7 AMPVs. Square boxes denote non-similar amino acid substitutions. Circles denote substitutions of similar amino acid residues.

4. Discussion

While embarking on preliminary studies concerned with making #8544 based modified vaccine, it was found that a FL copy of field strain #8544 itself would not lead to virus recovery in the Vero cell based RG system. Systematic substitution of Vaccine 1 unique sequences was used to investigate which #8544 sequence was preventing virus recovery by RG and replication in Vero cells. The investigation did not consider F sequence changes because a previous study had suggested its lack of involvement [9] and this was vindicated by the results presented here. Conversion of p#8544 by substitution of vaccine 1 sequence between genome positions 4874 and 6371 led to virus first being recovered. This implied that any of the differences between #8544 and vaccine 1 in that region, comprising mutations at 5055, 5140, 5929 or 6358, might be permissive of Vero growth. The coding mutations at 5055 and 5140 were considered likely to have greatest potential influence. However the non coding mutation occurring at 5929, in the SH-G untranslated region, could not be discounted because mutations in the same region in candidate vaccines highly related to Vaccine 1, had previously been shown to highly influence level of conferred protection [22]. The mutation of least probable significance was considered that at 6358 because the predicted amino acid coding remained unchanged. However even this could not be discounted because of the possibility of unrecognised alternative open reading frames. Simple sequence analysis of the relevant G gene showed genome position 6358 to reside within a potential 90 aa open reading frame, and if expressed, the mutation would lead to a predicted L to S substitution.

Substitution of vaccine 1 sequence into p#8544 between positions 2949 and 6416 led to virus recovery by RG whereas substitution of individual nucleotides at positions 5055, 5929 or 6358 did not. In contrast, substitution at 5140 led to virus clear CPE in first passaged material. The predicted effect of the 5140 coding change was to leave M2:1 unaffected but cause a threonine for isoleucine substitution in M2:2, a hydrophobic to polar conversion, potentially leading to a significant change in protein properties. It remained unclear whether the mutation acted by modification of the M2:2 to one more suitable for Vero growth or alternatively growth became possible simply because a functional M2:2 product was no longer present. It was hypothesised that T at residue 12 might be O link glycosylated and in turn lead to M2:2 changes facilitating Vero cells growth. However NetOglyc prediction software indicated that neither this residue nor any of the other S and T residues in the protein were likely to be glycosylated. To explore this situation further, p#8544 variants was modified by corrupting either the M2:2 first, or first and second, AUG start codons while leaving M2:1 coding unaltered. Both of these FLs readily yielded virus in the RG system, hence it could be concluded that for an #8544 FL, the presence of M2 second orf expression, prevents virus rescue.

It initially appears counterintuitive that the loss of M2:2 should facilitate virus replication because a previous study showed that M2:2 deletion in AMPV subtype A led to reduced replication [24]. However for AMPV subtype C, deletion of M2:2 from a Vero adapted virus led to largely normal replication in Vero cells but again much inhibited replication in turkeys [25]. Similarly a study of the related respiratory syncytial virus (RSV) showed that loss of M2:2 resulted in markedly inhibited growth in the tracheas of mice and cotton rats as well as Hela, HEp-2 and MRC 5 cells whereas in Vero cells, virus replicated efficiently and produced larger than typical plaque formations. The authors suggested this cell type restriction in an M2:2 deletion mutant may have been because the suggested normal role of M2:2 in assisting switching to virus replication, was substituted by Vero cell "host factors" not present in the other cells and tissues [26]. Interestingly in a previous AMPV study of vaccine 1, ability to replicate in avian tracheal organ cultures was conclusively shown to have been lost [13] and it was assumed that replication and ensuing immune priming was occurring in another unspecified location. In the light of the RSV findings, it appears possible that the loss of M2:2 function due to the 5140 mutation may have been responsible for this tropism shift in Vaccine 1.

There are contrasting views about the ability to use Vero cells for isolation of subtype A and B field AMPVs. In our group, direct isolation has proved impossible even when using a range of available Vero cell line sources. However some groups have had more success, notably if inoculated Vero cells are multiply serially trypsinised and split [27]. The reason for the increased isolation success is not clear but, as the presence of trypsin is the main differentiating feature, it may be that the effect of trypsin on either viral surface proteins or Vero cell proteins in the presence of virus, is influential. However given the excessive number of trypsin assisted passages required, it may be that all such isolations are in reality adaptations involving M2:2 mutation. It is interesting that only the Vero adapted viruses of seven sequenced in our current study contained M2:2 amino acid changes likely to affect the protein function. This is suggestive of the notion that Vero cell adaption of AMPVs require such mutations but clearly many more Vero adapted strains would need to be assembled or prepared, then sequenced to be able to justify such an assertion.

The study adds to the speculation as to which of the coding mutations, identified by sequencing [15], in the conversion of #8544 to vaccine 1 [7] were responsible for the observed attenuation. Coding mutations were limited to the F and M2 genes and, in the light of above discussions, it is possible that the 5140 mutation alone attenuated due to the tropism change already described [13]. Vaccine 1 did not replicate in avian trachea and it was deduced to be replicating, and presented to the immune system, in unspecified tissues. This might lead to the future prospect of rapidly producing AMPV vaccines from current field strains simply by M2:2 modification. This could be conveniently achieved by making a FL copy by modification of an existing FL, adding the M2:2 change, then recovering virus in the RG system [16]. This could be especially important in the context of the growing evidence of field strain evolution allowing avoidance of immunity from older vaccines [5,6]. However the role of the fusion protein also needs consideration. In another study focussing on induced protection in vaccine candidates, the substitution of the #8544 F gene into a

German isolated AMPV led to a chimera showing increased protection but also a small virulence increase [9]. While that chimera virus was much less virulent than #8544, it would probably have been greater, had G expression not been reduced due to SH gene end mutation. The role of F in the virulence of Vaccine 1 and AMPVs in general has not yet been fully investigated and this is likely to be a focus for our future studies and vaccine developments.

The foremost practical outcome of the study is that AMPV field virus # 8544 can be adapted to Vero cells by alteration of the M2:2 second open reading frame. This is a major step forward because the two major subtype A AMPV live vaccines available worldwide are empirical adaptations of the #8544 field strain, hence this finding will open the way to specific rational modification of #8544 for vaccine development purposes by Vero cell based reverse genetics. It might further offer a mechanism for Vero adaption and attenuation of non #8544 field strains.

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