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A comparison of AMPV subtype A and B full genomes, gene transcripts and proteins led to reverse genetics systems rescuing both subtypes.

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Abstract:	Avian Metapneumovirus (AMPV) infection of poultry causes serious disease in most countries and subtype A reverse genetic (RG) systems have allowed generation of viruses of known sequence, and proved useful in developments towards better control by live vaccines. While subtype B viruses are more prevalent, bacterial cloning issues made subtype B RG systems difficult to establish. A comparison of subtype A and B viruses was undertaken to assess whether subtype A RG components could be partially or fully substituted. AMPV subtype A and B gene end sequences leading to polyadenylation are reported for the first time, as well as several leader and trailer sequences. After comparing these alongside previously reported gene starts and protein sequences, it was concluded that subtype B genome copies would be likely to be rescued by a subtype A support system, and this assertion was supported when individual subtype A components were successfully substituted. Application of an advanced cloning plasmid permitted eventual completion of a fully subtype B RG system, and proved that all subtype specific components could be freely exchanged between A and B systems.

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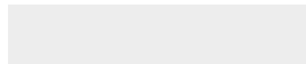

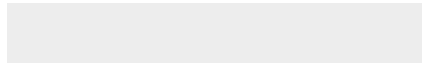

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10 **A comparison of AMPV subtype A and B full genomes, gene transcripts and proteins led to**
11 **reverse genetics systems rescuing both subtypes.**
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ABSTRACT

Avian Metapneumovirus (AMPV) infection of poultry causes serious disease in most countries and subtype A reverse genetic (RG) systems have allowed generation of viruses of known sequence, and proved useful in developments towards better control by live vaccines. While subtype B viruses are more prevalent, bacterial cloning issues made subtype B RG systems difficult to establish. A molecular comparison of subtype A and B viruses was undertaken to assess whether subtype A RG components could be partially or fully substituted. AMPV subtype A and B gene end sequences leading to polyadenylation are reported for the first time, as well as several leader and trailer sequences. After comparing these alongside previously reported gene starts and protein sequences, it was concluded that subtype B genome copies would be likely to be rescued by a subtype A support system, and this assertion was supported when individual subtype A components were successfully substituted. Application of an advanced cloning plasmid permitted eventual completion of a fully subtype B RG system, and proved that all subtype specific components could be freely exchanged between A and B systems.

INTRODUCTION

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

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

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

Comparison of complete genome sequences has shown that subgroups A, B and D are more related to each other than subtype C (Brown *et al.*, 2014) and another comparison of subtypes A, B and C showed subtype A and B to have the most similar genomes (Jacobs *et al.*, 2003). Subtypes A and B also appear to be most similar in their species specificity and behaviours in the field, hence live subtype A and B vaccines have been employed largely interchangeably to control disease in

98 commercial turkeys and chickens, albeit with an increasing bias toward subtype B. Cross protection
99 and antigenic studies have suggested that some protective and antigenic differences do exist
100 (Collins *et al.*, 1993; Cook *et al.*, 1993; Van de Zande *et al.*, 2000) and this highlighted the need for
101 a reverse genetics system to enable the generation of improved live subtype B vaccines, as well as
102 to understand other properties of this subtype.
103
104 A project to develop a subtype B reverse genetics system was initiated in our laboratory soon after
105 the subtype A development (Naylor *et al.*, 2004) but encountered problems. Also at a similar time
106 other groups were known to have initiated similar ventures yet no system was forthcoming. In our
107 case this was due to problems encountered while attempting to clone larger subtype B genome
108 sections into the plasmids previously found successful for cloning subtype A viruses. While N, P
109 and M2 genes could be readily cloned, the L gene and full genome proved impossible, as sequences
110 proved toxic even using the specialist tolerant cloning bacteria previously found adequate for
111 subtype A. This either led to the complete absence of clones, or clones containing major deletions,
112 often of several thousand nucleotides.
113
114 With a view to potentially utilising some of the available subtype A RG system components in the
115 development of a subtype B system, it was decided to investigate properties of subtype A and B
116 viruses likely to affect rescue and replication. Leader and trailer sequences essential for attachment
117 of the viral polymerase were determined and compared, as were those sequences recognised by the
118 viral polymerase in initiating and terminating the transcription of individual viral genes. The study
119 further compared protein similarities, especially for N, P, M2 and L which are all directly involved
120 in encapsidation, replication and transcription of the genome in a reverse genetics system. In most
121 cases we report for the first time the individual gene transcription stop signals for both subtype A
122 and B virus genes, as well as many previously unreported leader and trailer sequences. While many
123 gene stop sequences were predictable from available genome sequences, others were not, especially
124 where more than one termination like sequence was present at a gene end, as for example seen with
125 the M2 and G genes. When combined, results of these studies suggested that subtype A and B
126 reverse genetics systems might be able to recover full genome copies of the opposite subtype. Due
127 to the importance of AMPV subtype C in North America and elsewhere, comparison included an
128 established virus from that subtype.
129
130 During the investigation cloning attempts were continued and during these, a literature search
131 brought to our awareness a commercial plasmid pSMART that had permitted problematic regions
132 of an influenza virus genome to be successfully cloned (Zhou *et al.*, 2011). This was applied in
133 cloning the subtype B full genome and L gene. Finally a subtype B cloned genome was rescued
134 with either subtype A or B support components, hence this study includes report of the first AMPV
135 subtype B reverse genetics system. We also demonstrated the rescue of a subtype A virus using this
136 subtype B reverse genetics system.
137 .
138
139 **RESULTS**
140
141 **Determination and comparison of leaders and trailer sequences**
142
143 Determined leader and trailer sequences are give in Table 1 and sequence chromatograms in Figure
144 1. For reference, leader and trailer sequences from a previously published subtype C virus are
145 included in Table 1. For subtypes A and B, leaders or trailers sequences were always found to be in
146 agreement for viruses within the same subtype.
147
148 The leader sequences of subtype A and B viruses were identical for the first 12 nucleotides and
149 when compared to antigenomic trailer sequences, for subtype A they were identical for those first

150 12 nucleotides, whereas differences were found for subtype B. After position 12 similarities became
151 minimal.
152
153 For the trailer, an antigenomic sequence from nucleotides 13-21 GGCAUAAGU was detected in all
154 3 subtypes. For all 3 subtypes the remaining 18-24 nucleotides of the leader/trailer sequences up to
155 the N start/L end were mainly comprised of apparently random Us and As and there was no obvious
156 common sequence motif between the subtypes.
157
158 The 2 GGs normally assumed to be added to the virus leader due to use of a T7 promoter in RG
159 derived viruses were never detected.
160
161 **Determination and comparison of gene start and stop sequences**
162
163
164 Determined mRNA sequence chromatograms for each gene are shown in Figure 2. Gene start and
165 stop sequences for subtype A and B viruses are compared in Table 2 in genome sense (3'to 5') and
166 include sequences predicted from a previously determined published subtype C virus full genome
167 (accession number AY579780). All genes started with the sequence 3'CCCUGUUCA5' with the
168 exception of F and SH genes of subtype B which started with 3'CCCCGUUCA5'. All gene stop
169 signals started with UCA then had a variable sequence of generally 3 to 5 nucleotides after which
170 followed between four and seven Us (which became the polyA tail), with the exception of the
171 subtype A SH gene which had an 11 nucleotide separation but which still efficiently stopped
172 transcription and led to polyadenylation. In the case of Germany A virus, sequence changes within
173 this 11 nucleotide region led to absence of detectable monocistronic SH mRNA. This absence of
174 detectable SH gene transcription termination would be assumed to prevent downstream G
175 expression (Naylor *et al.*, 2007; Whelan *et al.*, 2004). Otherwise the subtype A and B transcription
176 stop sequences were very similar as shown in Table 3 with a consensus for subtype A of
177 UCAAU(A/U)A(A/U)UUUU and subtype B of UCAAUAU(A/U)UUUU.
178
179 **Comparison of viral protein sequences**
180
181 Details of nucleotide identities, together with amino acid sequence identities and similarities for
182 subtypes A, B and C are given for each gene in Table 4. Comparison of A, B and C sequences
183 confirmed that subtype A and B proteins were more closely related to each other than they were to
184 subtype C. Between subtypes A and B, those proteins expressed from transfected cloned DNA in
185 the reverse genetics system, N, P, M2 and L, had amino acid similarities of over 80%, and this was
186 also the case for M and F. In contrast when comparing either subtypes A or B to subtype C, the
187 similarity fell to approximately 79% in the case of the L gene. For the nonessential genes SH and G
188 (Naylor *et al.*, 2004), amino acid similarities between subtypes A and B were much lower at 60%
189 and 46% respectively and fell to approximately half those values when SH and G of either subtype
190 was compared to subtype C.
191
192 **Recovery of virus from AMPV full length copies**
193
194 Combinations of cloned genes and genomes from both A and B subtypes are given in table 5, which
195 shows that all combinations of subtype A and B components led to virus rescue.
196
197
198 DISCUSSION
199
200 Comparison of subtype A and B amino acid sequences of those proteins required for the RG system,
201 N, M2 and L, showed very high levels of amino acid identity and similarity while P had a lower

202 identity yet maintained 88% similarity. The fusion and matrix proteins were also highly similar.
203 While SH and G genes identities were much lower, these genes are not required for virus
204 replication in cell culture (Naylor *et al.*, 2004) or turkeys (Naylor *et al.*, 2010) so those differences
205 were not considered an impediment to virus rescue. The subtype C sequences were more different,
206 having polymerase identities and similarities with subtype A and B viruses of 64% and 79%
207 respectively. These data suggested that subtype A and B viruses might be recovered from subtype A
208 or B full-length genome copies using either subtype A or B support proteins. It is not clear whether
209 in spite of the greater differences found for the subtype C polymerase, subtype A and B reverse
210 genetics components might still recover virus from subtype C full length copies.
211
212
213 For similar viruses, the viral polymerase is known to recognise sequences in the leader and trailer
214 which play a role in transcription, replication and genome encapsidation. (Whelan *et al.*, 2004). The
215 leader sequences of AMPV subtypes A, B and C and antigenome trailer of subtype A were
216 identical for the first 12 nucleotides, whereas subtype B and C trailers had a 2 nucleotide mismatch.
217 Beyond nucleotide 12, virus leaders did not match their trailers and furthermore no common
218 sequence motif was seen when comparing between subtypes. In contrast within the antigenome
219 trailers of all three subtypes between nucleotides 13-21, a sequence of 3'GGCAUAAGU 5' was
220 found. When later the NCBI database was searched for all available equivalent sequences
221 (accession numbers HG934338 (subtype C, host duck), FJ 977568 (subtype C, host turkey),
222 AB548428 (subtype B, host chicken), AY 590688 (subtype C host turkey)) this same sequence was
223 always detected. While this sequence might be coincidental, it might also have some regulation role,
224 perhaps in the replication of the antigenome copy in subtype A, B and C viruses. However further
225 RG based studies would be required to substantiate such a hypotheses. But whatever the specific
226 role of the sequence, or the extreme 12 nucleotides of the leaders and trailer, the similarity across
227 subtypes would appear compatible with the notion of a subtype independent RG system.
228
229 Interestingly, while the use of a T7 promoter in the RG system would be expected to add two GG
230 residues to the start of the antigenome copy which would be expected to be incorporated into the
231 genome, and have sometimes been suspected of causing phenotypic differences between
232 recombinant and original virus from which the DNA copy has been prepared, these were never
233 detected. We therefore conclude that these are edited out at an early stage of the RG rescue. This is
234 a helpful practical observation because while the T7 promoter is very useful in RG systems, it is
235 sometime avoided because of this perceived implicit sequence addition.
236
237 A previously comprehensive minigenome investigation of gene start signal efficiencies showed that
238 the CCCUGUUCA was most efficient and that the variant sequence of CCCCGUUCA found on
239 subtype B SH and G proteins would be expected to reduce transcription of those genes (Edworthy
240 & Easton, 2005). The L gene transcription start sequences proved an exception and minigenome
241 studies showed a reduced transcription efficiency (Edworthy & Easton, 2005), as might be expected
242 for a gene coding a protein needed in smaller amounts. Surprisingly gene starts of the otherwise
243 more distantly related subtype C viruses (Brown *et al.*, 2014) like the subtype A viruses all used
244 CCCUGUUCA, but again with the exception of the L gene. Clearly lack of gene start differences
245 would means that gene start differences would not preclude a subtype independent RG system for
246 AMPV.
247
248 Transcription stop sequences had not been previously reported for most AMPV genes. In general
249 the sequences found for AMPV subtype A and B were in agreement with those found previously for
250 respiratory syncytial virus (Harmon *et al.*, 2001). Nonetheless, a study of seven recombinant
251 subtype A viruses, each containing a GFP reporter gene at different intergenic regions had shown
252 that GFP expression did not follow the accepted model and suggested that inefficient genome stop
253 sequences may have been playing a role (Falchieri, 2012), as had already been found to affect

254 protection induced by candidate vaccines only differing in the their SH gene ends (Naylor *et al.*,
255 2007). Similarly in the current study it proved impossible to detect monocistronic SH mRNA in a
256 German field strain which implies that the downstream G gene would be unlikely to be expressed,
257 and may well help explain why in a previous study, the deletion of this G gene from the same virus
258 only marginally reduced its protective capacity (Naylor *et al.*, 2010). Nonetheless stop sequence
259 differences between subtypes were not generally greater than those within subtypes, hence
260 supported the notion of a subtype independent RG system.
261
262 The above data taken as whole suggested that for an AMPV RG system subtype A and B
263 components might be fully interchanged. This proved the case because when subtype B
264 components became available they proved able to be substituted for subtype A components in the
265 RG systems – and once a fully subtype B RG had been produced, both subtype A and B full length
266 genome copies were shown to efficiently produce virus when using either subtype A or B support
267 proteins. This indicates that the viral polymerase of either subtype is able to attach to the leader and
268 trailer, to recognise gene start and stop sequences, and that the key viral protein genes shared
269 sufficient functional similarity to support rescue. It remains uncertain as to whether subtype A/B
270 components might be able to recover a full length subtype C copy, though this could easily be tested
271 through collaboration between groups in possession of the different RG systems.
272
273 As a more practical point, the cloning of genome copies in bacterial plasmids offer considerable
274 flexibility when compared to alternatives more able to handle difficult sequences such as cloning
275 into bacterial artificial chromosomes or other larger viruses such as fowlpox or vaccinia. In this
276 study the previously recognised ability of pSMART to accept influenza virus genome segments has
277 been extended to included the full genomes of an AMPV genome exhaustively proven very difficult
278 to otherwise clone. It would interesting to know the limits of this approach and perhaps explore
279 potential with larger viruses such as coronaviruses.
280
281
282 METHODS
283
284 **Viruses**
285
286 The subtype A (Germany A) virus used to create the first AMPV reverse genetics system was
287 isolated in Germany in the 1990's (Naylor *et al.*, 2004) and was later tested in vaccination studies
288 (Naylor *et al.*, 2010). Other subtype A field viruses sequenced for gene sequence comparison were
289 #8544(Jones *et al.*, 1986), Italy 259 (Cecchinato *et al.*, 2010), UK 3B (Mcdougall & Cook, 1986),
290 CVL 14-1 (Collins & Gough, 1988) and UK CP/1 (Jones *et al.*, 1991); and commercial live
291 vaccines Poulvac TRT, Nobilis TRT and Turkadin (discontinued).
292
293 The subtype B virus used to create the first AMPV subtype B reverse genetics system was a vaccine
294 strain derived from UK strain 11/94. Subtype B field viruses sequenced for gene sequence
295 comparison were Italy 205 and 240 (Cecchinato *et al.*, 2010), France 147 and 38 (Cook *et al.*,
296 1993), Netherlands 27 (Cook *et al.*, 1993), Italy 16-91(Cook *et al.*, 1993); and commercial live
297 vaccines Nemovac, Aviffa and Nobilis Rhino CV.
298
299 **Determination of leader and trailer sequences**
300
301 Leader and trailer sequences were determined by 3'RACE on the genome and antigenome
302 respectively using a previously described method (Brown *et al.*, 2013). Viruses sequenced were
303 #8544, Poulvac TRT, Italy 240, RhinoCV and Nemovac. Subsequently leader and trailer sequences
304 of recombinant rescued viruses (recombinants of Germany A, Fort Dodge vaccine, Rhino CV) were
305 determined.

306
307 **Determination of 3' termini sequence of subtype A and B AMPV mRNAs**
308
309 Virus messages were amplified by RT-PCR using the technique previously described in our
310 laboratory (Brown *et al.*, 2011). Briefly RT was performed with a primer containing 20 Ts
311 followed by an adaptor sequence at its 5' terminus. This was amplified by PCR using 2 primers, one
312 within in the respective genes and one matching the adaptor. These PCR products were sequenced
313 towards the polyA tail using the same gene specific primers.
314
315 **Determination of viral gene sequences and their comparison.**
316
317 Sequences of subtype A and B virus genes were determined by sequencing of PCR amplified
318 genome sections, as described in previous studies (Brown *et al.*, 2011; Cecchinato *et al.*, 2010;
319 Naylor *et al.*, 2004; Naylor *et al.*, 2007). Using Bioedit, nucleotide sequences aligned and inter-
320 subtype identities calculated, then sequences were translated to allow predicted amino acid
321 identities and similarities to be calculated.
322
323
324 **Construction of subtype B reverse genetics system**
325
326 Subtype B support protein genes N, P, M2 were cloned into the same plasmids as had been used
327 previously in the subtype A rescue system (Naylor *et al.*, 2004).
328
329 To construct the subtype B genome copy, sections were amplified by high fidelity RT-PCR using
330 primers introducing Xho1/Sal 1 RE sites at positions shown in Figure 3. The approach was
331 essentially that previously employed in constructing our subtype A reverse genetics system (Naylor
332 *et al.*, 2004). Because of previous stability issue of copied DNA in cloning bacteria, sections were
333 sequentially cloned into the pSMART LC Kan (Lucigen) as shown in Fig 3 to produce a full
334 length genome copy preceded by the T7 promoter and followed by the Hepatitis delta virus
335 ribozyme (HDVR).
336
337 For the L gene, because of cloning stability issues with the original plasmid used to clone the
338 subtype A L, it was copied by hi-fidelity PCR from the cloned full subtype B genome to include the
339 pSMART LC Kan sequence . This was ligated and cloned.
340
341 **Recovery of viruses**
342
343 Vero cells infected with a fowlpox recombinant virus expressing T7 polymerase were transfected
344 initially with a cloned subtype A genome, together with subtype A support protein genes, and
345 cloned subtype B support protein genes as they became available, using Lipofectamine 2000, under
346 the same conditions and concentrations previously used for subtype A rescue (Naylor *et al.*, 2004).
347 Subsequently the cloned subtype B genome replaced the subtype A genome. Eventually subtype B
348 components entirely replaced those from subtype A. In addition a subtype B genome copy was used
349 with only subtype A components. Details are given in Table 5.
350
351
352
353
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Table 1

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

AMPV subtype	Leader and complimented trailer sequences for subtype A, B and C viruses
A leader	3' **UGCUCUUUUUUUUGCAUAAAUUCGUC**...........N start 5'
A trailer[1]	3' **UGCUCUUUUUUUUUGGCAUAAGUAGU**...........L stop 5'
B Lead	3' **UGCUCUUUUUUUUGCGUAAGUUCAG**...........N start 5'
B trailer[1]	3' **UGCCGUUUUUUUGGCAUAAGUUAU**...........L stop 5'
[2]C leader	3' **UGCUCUUUUUUUUGCGUAUAUUCUG**...........N start 5'
[2]C trailer[1]	3' **UGCCGUUUUUUUGGCAUAAGUAGG**...........L stop 5'

[1] antigenome sequence

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

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Table 2

Transcription start sequences and determined transcription stops for subtype A and B viruses

Gene	AMPV subtype	Sequence from transcription start to subsequent transcription start
N	A	CCCUGUUCAGUUUU -ORF+NCGE- UCA UUAA[2]UUUUUUUUAUA
	B	CCCUGUUCAUUUU -ORF+NCGE- UCA UUAA[2]UUUUUAAG
	C[1]	CCCUGUUCACUUU -ORF+NCGE- UCA UUAAUUUUUUUUAUA
P	A	CCCUGUUCAUUGU -ORF+NCGE- UCA AUAC[2]UUUUUUA
	B	CCCUGUUCACUUU -ORF+NCGE- UCA AUAC[2]UUUUUUA
	C[1]	CCCUGUUCAGUUU -ORF+NCGE- UCA AUUAUUUUUUG
M	A	CCCUGUUCAGUUU -ORF+NCGE- UCA GUUA[2]UUUUUUAA
	B	CCCUGUUCAUUUG -ORF+NCGE- UCA AAUUA[2]UUUUUUAUA
	C[1]	CCCUGUUCACCUU -ORF+NCGE- UCA GUUCUAUUUGUGUCUCUCAUGUGAAUGGUUUAGUGUCAUU GUUAAAGCAAAAAUUGGGAGAGUAUCAAUAAUGGAUCGAACUAUAAUAAAUCUUUUUUAA
F	A	CCCUGUUCAUCC -ORF+NCGE- UCA AUAAA[2]UUUUAA
	B	CCCCGUUCAUUU -ORF+NCGE- UCA AUGUA[2]UUUUUUCA
	C	CCCUGUUCACUUU -ORF+NCGE- UCA AUGAUUUUUUUAA
M2	A	CCCUGUUCACUUC -ORF+NCGE- UCA AUUAA [2]UUUUGGUUAAUUCGAUAUUCAGGUUUUUUCCCA
	B	CCCUGUUCAUUUC -ORF+NCGE- UCA AUAUA[2]UUUUUGUUAACUCGUGGGGGGGGCUUUUUUCUA
	C[1]	CCCUGUUCACUUC -ORF+NCGE- UCA AUUAUUUUUUUAA
SH	A	CCCUGUUCAGUAU -ORF+NCGE- UCA UAAUAAAUUAA[2]UUUUUCUUUCCAG
	Germany A	CCCUGUUCAGUAU -ORF+NCGE- UCA UAAUAAAUAAAUGUUUCUUUCCAG did not stop
	B	CCCCGUUCAGUUC -ORF+NCGE- UCA AUUAA[2]UUUUAGUCUUCUG
	C[1]	CCCUGUUCAGUUG -ORF+NCGE- UCA AUAAAUUUUUAGUACUUAUACAGACCUGUCACGGUUCCGGUUC UUUUUGGUUGUGCUCUUGUCCACUAGGUUACUAAUUUUUGCUAGUCUCUUCCUUUUUG
G	A	CCCUGUUCAUAGAGU-ORF+NCGE- UCA AUUGA[2]UUUUUACUUGUGUAUAUAUAGACUAUUAUUUUU UUGUGUAGUCUAUCAGAUUUUGUUAAUUUUCUUACUUUUGU
	B	CCCUGUUCAUAGGUC-ORF+NCGE- UCA GUUA[2]UUUUUCAUUGGAAAGUGUAGAUUUUAUUUCGUUUU UCUUCUUUUUUCUUCUUUCUUCCUUUCUUUCUUCUUCUUAUCGUGUGUUGUCUUUCCU
	C[1]	CCCUGUUCAGUUG -ORF+NCGE- UCA AUUAAUUUUUCUU
L	A	UCCUGGUUA -ORF+NCGE- UCA AUUA[2]UUUUU to Trailer
	B	CCCUGGUUA -ORF+NCGE- UCA AUA[2]UUUUUU to Trailer
	C[1]	CCUGGUUCA -ORF+NCGE- UCA AUAAAUUUUU to Trailer

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

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Table 3

Determined Consensus gene stop signals for subtype A and B viruses, with predicted subtype C sequences based on database reference.

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

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

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Table 4

Nucleotide identities, and predicted amino acid identities and similarities, comparing AMPV subtypes A, B and C.

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

Shading denotes greater than 80% identity/similarity

[1] nucleotide identity
[2] amino acid identity
[3] amino acid similarity

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Table 5

Combinations of RG components used in virus rescue attempts

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

Figure
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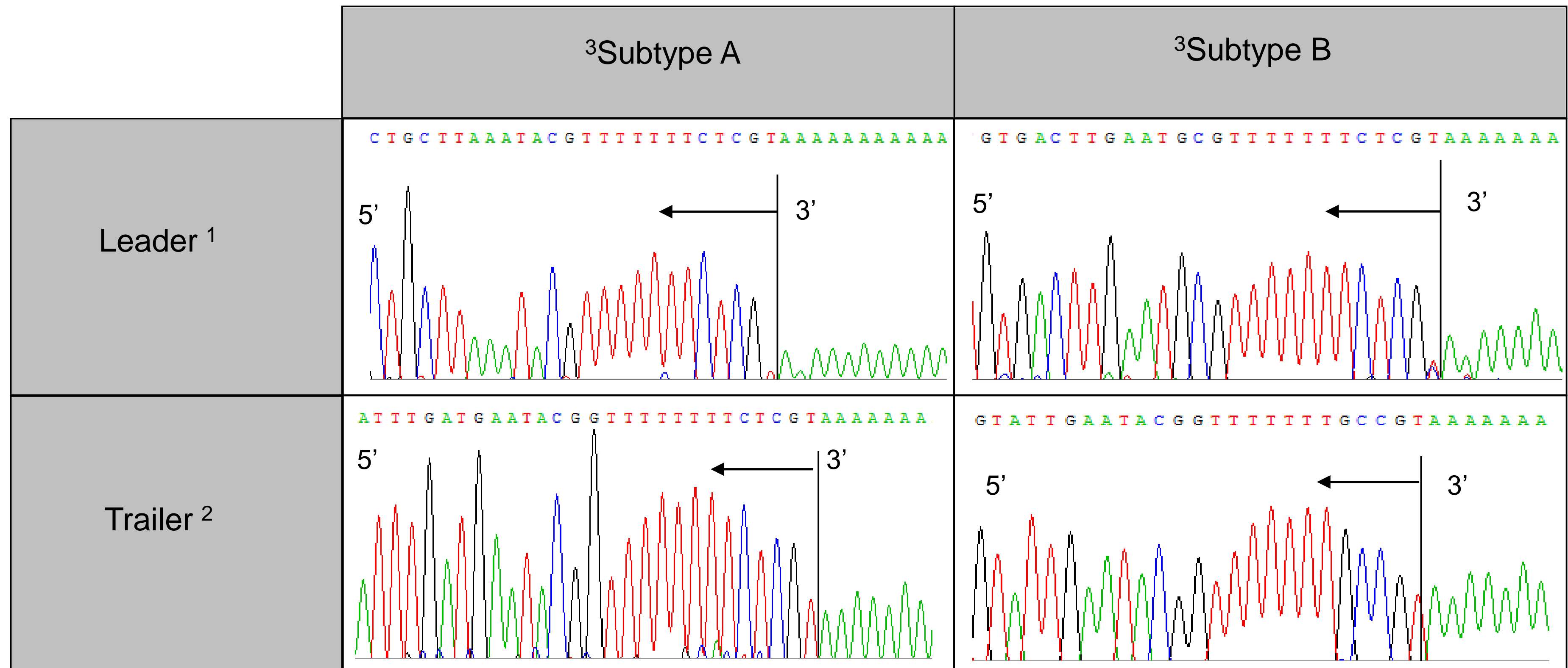

[1] polyadenylated DNA copies of genomic sense leader

[2] polyadenylated DNA copies of antigenome sense trailer

[3]Sequence common for all viruses of this subtype

Figure

Figure 2

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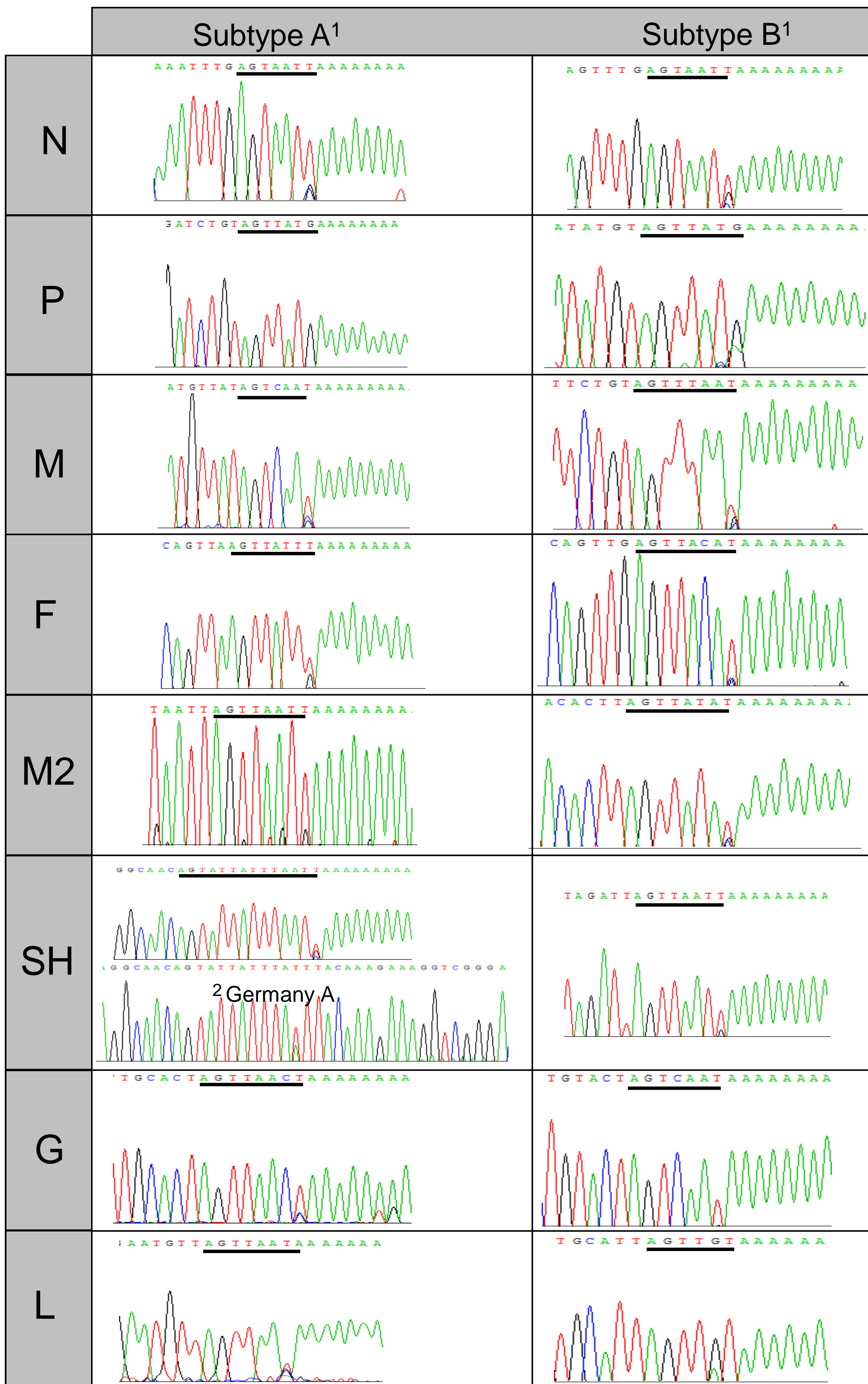

[1] Common terminal sequence found in all viruses sequenced except for subtype A, SH gene

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

Underlined sequences are common between all viruses sequenced except subtype A, SH gene

Figure

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