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Laconi A, Catelli E, Cecchinato M, Naylor CJ. 2019. Two similar commercial live attenuated AMPV vaccines prepared by random passage of the identical field isolate, have unrelated sequences. *Vaccine*, 37:2765-2767.

The final published version is available online at: <https://doi.org/10.1016/j.vaccine.2019.04.036>

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1 **Two similar commercial live attenuated AMPV vaccines prepared by random passage of the**
2 **identical field isolate, have unrelated sequences.**

3
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12
13 Article type: short communication

14 Keywords: vaccine, avian metapneumovirus, attenuation, farm vaccine detection

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23
24 **Abstract**

25
26 Since late '80s Avian metapneumovirus subtype A causes sufficient disease in Europe for
27 commercial companies to have started developing live attenuated vaccines. Here, two of those
28 vaccines were fully consensus sequenced alongside their progenitor field strain (#8544). Sequences
29 comparison shows that the attenuation of field strain #8544 was associated with no common
30 substitutions between the two derived vaccines. This finding suggests that the attenuation of field
31 viruses via serial passage on cell cultures or tissues is the result of a random process, rather than a
32 mechanism aiming to achieve a specific sequence. Furthermore, field vaccination strategies would
33 greatly benefit by the unambiguous vaccine markers identified in this study, enabling a prompt and
34 confident vaccines detection.

35

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36 **1. Introduction**

37

38 Avian metapneumovirus (AMPV) has caused disease and economic losses in unprotected
39 commercial turkeys and chickens for at least four decades. In general the virus has been controlled
40 by the preparation and use of highly effective live attenuated vaccines, made by random passage of
41 a number of AMPV field isolates in various cells or tissues [1,2]. In a small minority of
42 circumstances, AMPV vaccines have been ineffective, and disease has sometimes been attributed to
43 the use of vaccine subtype not matching the AMPV subtype in circulation [3]. On other occasions
44 vaccine has been shown to cause disease by mutating back to a virulent state in the vaccinated birds,
45 though fortuitous events and much background work was needed to prove this [4,5]. For some years,
46 our laboratory has recognised the need for nucleic acid sequence markers for live attenuated poultry
47 vaccines to enable vaccine tracking on farms. To that end, we have successfully identified several
48 markers for live AMPV and infectious bronchitis virus (IBV) commercial vaccines and these in turn
49 are now being used to monitor those viruses in commercial flocks internationally [4,6,7].
50

51 To be able to identify reliable sequence markers for a particular live attenuated vaccine, it is
52 necessary to sequence both the vaccine and the field virus used in its preparation, ideally in their
53 entirety. Comparison then reveals the mutations generated during the attenuation process.

54 Regarding the approximately fourteen kilobases AMPV genome, very roughly ten mutations have
55 been found associated with the attenuation process [4,7], whereas for the roughly 28 kilobases IBV
56 genome, the number of mutations is approximately doubled [6]. Finally, once mutations markers
57 are identified, they are ideally compared to field strains present prior to a vaccine's introduction, so
58 as to eliminate the highly unlikely possibility that the same mutations might have been generated by
59 other chance mechanisms.
60

61 In the current study we report an unusual vaccine marker study whereby two commercial subtype A
62 AMPV vaccines had been prepared from the identical progenitor field strain. In the late 1980s,
63 AMPV (then named TRTV) was causing severe disease in the UK. Morbidity and mortality were
64 crippling the turkey industry and there was an urgent need to produce protective vaccines. Very few
65 virus isolates of this newly discovered virus were available and as a result a large UK based
66 commercial turkey breeding company made their recently isolated field isolate #8544 [8,9]
67 available to two different commercial vaccines producers. Both companies proceeded to attenuate
68 the virus and release vaccines, both of which remain widely used throughout the world. Our group
69 has previously described the generation of one of these vaccines [2,10] and is here named vaccine 1,
70 but in the other case (vaccine 2), details of the attenuation procedure remains unreported. In this
71 study, we have sequenced both vaccines in their entirety and compared them to determine vaccine
72 markers. We have gone on to consider the implications of the marker patterns with respect to the
73 justification of our vaccine marker approach, as well to consider the possible significance of the
74 mutations discovered.
75

76 **2. Materials and methods**

77

78 **2.1 Viruses**

79 Field virus #8544 was isolated in turkey embryo tracheal organ culture (TOC) from a field outbreak
80 [9]. To produce vaccine 1, field isolate #8544 virus was attenuated by two passages in chick embryo
81 liver cells then 20 passages in Vero cells. Vaccine 2 was produced from the same progenitor; while
82 details of cells and tissues used have not been disclosed by the company concerned, Vero cells may
83 also have been involved. Notably, prior master seed preparation (MSV), both the vaccines had been
84 cloned by plaque purification following the attenuation passages on cells.
85

86

87

87 **2.2 Viral RNA extraction, RT-PCRs and sequencing**

88 Field virus #8544, vaccine 1 and vaccine 2 were sequenced using the method previously reported
89 [11]. Briefly, RNA was copied to full-length cDNA then amplified in three overlapping sections to
90 cover the complete genome, then sequenced by Sanger sequencing using oligonucleotide primers
91 matching known conserved regions. Where vaccines and progenitor consensus sequences differed,
92 the locations were sequenced again, starting from newly isolated RNA.

93

94 **2.3 Sequences analysis and comparison**

95 Chromatograms were analysed using the program Chromas
96 (<http://technelysium.com.au/wp/chromas/>) and sequences aligned using BioEdit
97 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Open Reading Frame (ORF) prediction was
98 carried out using ORFfinder program (<https://www.ncbi.nlm.nih.gov/orffinder/>). Predicted amino
99 acids sequences were aligned using GeneRunner (<http://www.generunner.net/>).

100

101 **3. Results and discussion**

102

103 A comparison of virus sequences is depicted in table 1. Since their commercial release in the early
104 1990s, both #8544 derived vaccines have been used to successfully protect many millions of
105 chickens and turkeys on farms throughout the world. It is striking that despite the two vaccines do
106 not share any of the same attenuating mutations, they showed, as reported in the two registration
107 dossiers, a similar if not identical attenuated phenotype in vivo. Furthermore, after release of both
108 vaccines, veterinarians in the UK and in continental Europe reported throughout the years their
109 similar behaviour in the field. The attenuation of #8544 to produce vaccine 1 was associated with
110 ten mutations, while vaccine 2 had seven mutations. Remarkably, none was the same. One broad
111 sequence similarity was that for both vaccines, mutations within coding regions occurred between
112 the F gene and the trailer end of the genome. For vaccine 1 genes altered were F, M2, G and L and
113 for vaccine 2, F, SH and L. However, this similarity may be purely coincidental.

114

115 A sometimes-cited criticism of use of nucleotide vaccine markers determined by progenitor/derived
116 vaccine comparisons is that such identical mutations might arise randomly and spontaneously in the
117 field. If truly random, simple calculation can show this possibility to be effectively zero. However,
118 it might be hypothesised that certain specific field conditions select subpopulations sharing these
119 same vaccine markers. While impossible to absolutely refute this possibility, the fact the two
120 independent passages of #8544 did not produce any common mutations goes some way.
121 Furthermore, we have never met an instance where detection of virus of apparent vaccine origin
122 could not be associated with application of vaccine somewhere within the environment of potential
123 spread. In our opinion, it is safe to conclude that any field virus found to contain two or three of the
124 identified vaccine markers for a particular vaccine will have derived from that vaccine, whether
125 having been applied on that farm or having spread to the farm via previous vaccinations at another
126 location [5].

127

128 The coding mutation differences in each vaccine appear to show that the mechanisms of attenuation
129 were different in each. For vaccine 2, three out of five coding mutations occurred in the L gene
130 which codes for the viral polymerase. Interestingly our previous study concerning causes of
131 reversion to virulence of vaccine 1 identified a single mutation in the L gene as responsible [12].
132 Thus, it is possible that attenuation of vaccine 2 was due to L gene changes. In contrast, the
133 attenuation of #8544 to produce vaccine 1 in the first place involved no L mutation. Taking as a
134 whole, this shows that mutations in a number of AMPV genome regions may lead to attenuation,
135 rather than via a single mechanism.

136

137 However, the coding fusion protein mutations found in vaccine 1 at nucleotide 3825 and in vaccine
138 2 at nucleotide 3822 may be related. Both mutations occur within a region spanning amino acids
139 293 to 296 of the fusion protein, whereby the sequence of charged amino acids RKEK in #8544 are

140 converted to RKKK in vaccine 1 and REEK in vaccine 2. Furthermore, during previously
141 unreported commercial vaccine studies, we found two possibly related points of interest. When
142 vaccine 1 reverted to virulence in experimental conditions, RKKK mutated back to RKEK,
143 although in association with other mutations. Secondly when reverse genetics was employed to
144 modify this region in a promising candidate vaccine, it was found that in general virus viability
145 required four charged amino acids in this region, though interestingly it could also be substituted by
146 GGGG. Since that work other groups have reported the importance of amino acid 294 in inducing
147 low pH membrane fusion in both avian and human metapneumovirus [13,14]. Hence, it is not
148 impossible that this region was involved in the attenuation of both vaccines 1 and 2 and it should be
149 considered a region worthy of further investigation.

150
151 Vaccine 1 was produced primarily by passage of #8544 in Vero cells as previously reported [2].
152 Throughout the process, some of these passages were tested as vaccine candidates. Clearly, it would
153 be of interest to go back and sequence those intermediate passages. However, because sequencing
154 of vaccines 1 and 2 has taken place some considerable period after the production of vaccine 1 in
155 the early 1990s, we no longer have access to those intermediates; hence, the possibility of
156 correlating specific mutations to loss of virulence has unfortunately been lost.

157
158 In conclusion, our study shows that production of similar empirical vaccines by serial passage is a
159 random process unrelated to the achievement of a particular final sequence. Furthermore, the un-
160 relatedness of determined vaccine 1 and 2 sequences strongly suggests mutation patterns to be
161 unique to each vaccine and thus the vaccine markers identified can be used with confidence to
162 identify AMPV vaccines and vaccine virus derivatives in the field.

163 164 **References**

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211 Table 1: Nucleotide substitutions arising during the attenuation of #8544 to yield vaccine 1 and
 212 vaccine 2. Shading denotes the amino acid changes occurred.

Position	Gene	#8544		Vaccine 1		Vaccine 2	
		Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
2941	UTR	G	NA	A	NA	G	NA
3029	F	T	S	T	S	C	S
3553	F	T	V	C	A	T	V
3822	F	A	K	A	K	G	E
3825	F	G	E	A	K	G	E
4100	F	G	E	G	E	A	E
5055	M2-1	A	K	G	R	A	K
5140	M2-1	T	N	C	N	T	N
5140	M2-2	T	I	C	T	T	I
5702	SH	G	C	G	C	A	Y
5929	UTR	A	NA	G	NA	A	NA
6358	G	T	L	C	L	T	L
8122	L	C	R	C	R	A	Q
8257	L	C	A	C	A	T	V
9591	L	A	N	A	N	G	D
10022	L	A	V	G	V	A	V
11624	L	T	Y	C	Y	T	Y