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

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## Abstract

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

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## 1. Introduction

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

To be able to identify reliable sequence markers for a particular live attenuated vaccine, it is necessary to sequence both the vaccine and the field virus used in its preparation, ideally in their entirety. Comparison then reveals the mutations generated during the attenuation process. Regarding the approximately fourteen kilobases AMPV genome, very roughly ten mutations have been found associated with the attenuation process [4,7], whereas for the roughly 28 kilobases IBV genome, the number of mutations is approximately doubled [6]. Finally, once mutations markers are identified, they are ideally compared to field strains present prior to a vaccine's introduction, so as to eliminate the highly unlikely possibility that the same mutations might have been generated by other chance mechanisms.

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

## 2. Materials and methods

### 2.1 Viruses

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

### 2.2 Viral RNA extraction, RT-PCRs and sequencing

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

### 2.3 Sequences analysis and comparison

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

## 3. Results and discussion

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

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

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

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

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

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

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

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

| | | #8544 | | Vaccine 1 | | Vaccine 2 | |
|---|---|---|---|---|---|---|---|
| **Position** | Gene | 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 |