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Molecular Characterization of Vaccine-Derived Mutants of Avian Metapneumoviruses Isolated from Turkeys in Egypt

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Abstract: Avian metapneumovirus (aMPV) causes acute respiratory infection in turkeys with sudden onset and rapid spread in the infected flocks. Vaccinated flocks frequently showed clinical signs typical like the non-vaccinated flocks in Egypt, this situation suggests a virulent vaccine virus was circulating in the field. Oropharyngeal and nasal sinus swabs were collected from 100 turkeys from 10 commercial farms with mild to moderate respiratory signs. Virus isolation and propagation was done in SPF embryos of chicken eggs via yolk sac inoculation and further passed in Vero cells. RT-PCR for detection of aMPV was done on field samples as well as on virus isolates. Genotyping of viruses was done through RT-PCR targeting Fusion (F) and the surface Glycoprotein (G) genes and partial sequencing of PCR products was used for genetic and phylogenetic analyses. The results were compared with two live attenuated commercial vaccines commonly used in the field. Analysis of nucleotide and amino acid sequences for F and G genes indicated that they were very closely related to each other and belonging to B subtype of aMPV isolated from Europe. The Egyptian viruses were very close to VCO3 vaccine strain, indicating their possible origin. Phylogenetic analysis indicates clustering of the Egyptian field viruses with European strains of B subtype. These results support that they may have a common progenitor from vaccine strains and suggesting evidence of reversion of attenuated vaccine virus to virulence. This is the first report describing the emergence of vaccine-derived mutants from the B subtype aMPV in broiler turkeys in Egypt.

Keywords: Avian Metapneumoviruses, Vaccine-Derived Mutants, Molecular Characterization of F and G Genes, aMPV in Egypt

Introduction

Avian metapneumovirus (aMPV), former was described as Turkey Rhinotracheitis (TRT) in turkeys and Swollen Head Syndrome (SHS) in chickens (Gough, 2003). The aMPV is an important pathogen causing respiratory disease and egg drops in chickens and turkeys. It was first described in South Africa in 1978 (Buys *et al*., 1989) and subsequently several outbreaks followed in the early 1980s in continental Europe (Giravd *et al*., 1986; Hafez *et al*., 2000). The aMPV causes an acute respiratory tract infection both in turkeys and in chickens with a sudden onset and rapid spread through flocks (Anon, 1985). Clinical signs are often mild, but in the presence of secondary bacterial infections the disease can be very severe and even fatal.

The genome contains eight genes present in the same order shown in the other metapneumoviruses, 3-N-P-M-F-M2-SH-G-L-5 and encodes 9 proteins, including Nucleoprotein (N), Phosphoprotein (P), matrix protein (M), Fusion protein (F), second matrix protein (M2), small hydrophobic protein (SH), surface Glycoprotein (G) and a viral RNA-dependent RNA polymerase (L). All aMPV genes encode one viral protein, except the M2 gene, which encodes two proteins; M2-1 and M2-2 (Tanaka *et al*., 1995; Sugiyama *et al*., 2010). Although pneumoviruses have F protein that upholds cell fusion, these viruses do not have hemagglutination property or neuraminidase activity in their G attachment protein and that distinguishing them from the other paramyxoviruses (Collins and Gough, 1988). The F and G proteins are the two major antigens of aMPV, where the G protein is

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known to be the most variable protein in human respiratory syncytial virus, which is the prototype of pneumovirus (Johnson *et al*., 1987), the F protein is one of the major immunogens expressed on the surface of pneumoviruses and so it is important in inducing protective immunity (Yu *et al*., 1994).

Avian metapneumoviruses have been classified into four subtypes, including A, B, C and D based on nucleotide sequence analysis and neutralization with monoclonal antibodies (Cook *et al*., 1993; Bayon-Auboyer *et al*., 2000). Many countries reported different subgroups of aMPV, mainly A or B, like Israel, Mexico, Morocco, Jordan, Brazil, Japan and many European countries (Hafez *et al*., 2000; OIE, 2008). The aMPV has been circulating globally since 1978, reported in the United States from a commercial turkey farm in Colorado in 1996 and subsequently in Minnesota (Cook *et al*., 1999). Few reports described the incidence and distribution of aMPV in Egypt; however, it caused serious problems in turkey production and affected the performance of turkey pouts and chickens (Collins and Gough, 1988; Mahmoud *et al*., 2008). As aMPV causes a considerable economic loss, several attempts have been made to develop a live attenuated vaccine (Cook *et al*. 1989a; 1989b; Williams *et al*., 1991). Vaccination has been used to protect flocks from virus infections to a certain extent (Buys *et al*., 1989).

Nucleotide sequence analysis of the G gene has been used for differentiating subtypes A and B and RT-PCR assays directed to the F and M genes are subtype specific and useful for detection of aMPV when subtype identity of the virus is known (OIE, 2008).

This work describes the molecular characterization of Avian metapneumoviruses isolated from infected turkey flocks in Egypt from 2008 to 2009 using partial sequencing of the (F) gene and the (G) gene and for differentiation between field and vaccine strains that was commonly applied in the field.

Materials and Methods

Samples

Oropharyngeal and nasal sinus swabs were collected from 100 turkeys from 10 commercial farms sourced from 3 Egyptian Governorates (Giza, Beni-Suif and Cairo) from 2008 to 2009 and named as Giza-1-5, 9, 10, Beni-Suif-6, 7 and Cairo-8. These flocks were vaccinated with different regimens, for example, 2 flocks (Giza-1 and Beni-Suif-7) with 2 vaccines (one day old live spray and inactivated combined ND+TRT s/c at 45 days) and two flocks (Giza-3, 4) with 3 vaccinations (1 killed then 2 live) and 1 non vaccinated flock (Cairo-8). Other flocks were vaccinated with at least one live attenuated vaccine.

The symptoms began from 10 days up to 2 months post last vaccination in vaccinated flocks and ranged from mild to moderate respiratory distress, facial edema and nasal discharge with sinusitis occasionally. The swabs were pooled from each farm (10swabs/pool) and immersed in Phosphate Buffer Saline (PBS) with antibiotic (Penicillin 0.5 U/ml and Streptomycin 0.5 mg/ml) then centrifuged at 1000 x*g* for 10 min. The supernatant were passed through a 0.450 µm membrane filter and kept at -80°C for virological and molecular diagnosis.

Vaccines: Two live attenuated commercial vaccines were used, Aviffa® and Nemovac®. They were used for sequence comparison between field and vaccine strains. The vaccine strain (VCO3/60616 B subtype, accession number: AB548428) was used for sequence comparison.

Virus isolation and propagation: The pooled swabs from the 10 cases were inoculated in SPF embryos of chicken eggs via yolk sac route at 5-7 days old embryos and further passed in Vero cells for two successive passages using Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum containing penicillin (0.5 U/ml) and streptomycin (0.5 mg/ml).

RT-PCR and Genetic Analysis

The B subtype strain UK/8/94 (GenBank Accession number Y14294) was used as the standard for comparisons (Juhasz and Easton, 1994; Naylor *et al*., 1998).

RNAs were extracted from oropharyngeal and nasal swabs from each case for direct detection of aMPV and also to confirm virus isolation using RNA Extraction kit (QIAmp Viral RNA Mini kit, Cat. No. 52904, Qiagen, Valencia, Calif, USA). Reserve Transcription (RT) was performed using RT random hexamer primers or G6-primer (Naylor *et al*., 1998) and AMV reverse transcriptase (Roche, Cat. No.13490320) according to the manufacturer's instructions, the RT reactions were done using 5 µL of purified RNA in a 20 µL reaction volume and this step was carried out on thermal cycler (T3, Biometra, Germany) with a thermal profile of 42°C/30 min then 85°C for 5 min.

Partial Fusion (F) and attachment (G) genes of aMPV were amplified by PCR with primers designed from consensus sequences of aMPV as follow: Polymerase Chain Reaction (PCR) was done in a final volume of 25 µL using PCR kit (ABgene-Thermo, UK) and 2 µL of gene specific primers for G and F genes (Table 1) and 5 µL of cDNA. The RT-PCR for aMPV was based on sequences of the G genes of A and B subtype viruses (Juhasz and Easton, 1994). The oligonucleotide, G6-, for priming the RT reaction, had sequence common to both subtypes and was used in the first PCR with oligonucleotide G1+, consequent to the beginning of the gene, that able to hybridize with RNA of both virus subtypes. The 444 base pairs (bp) product was used in a nested PCR with oligonucleotide G5 and able to hybridize to RNA of both subtypes with oligonucleotides G8+ A, specific for A subtype or G9+B, specific for B subtype, generating cDNAs of 268 and 361 bp, respectively (Naylor *et al*., 1997).

Table 1. Primers used for F and G genes of aMPV

Oligonucleotide	Sequence	Gene	Position	Reference
G6-	CTGACAAATTGGTCCTGATT	G	422 to 441	Juhasz and Easton (1994)
G5-	CAAAGA A/GCCAATAAGCCCAG	G	401 to 419	
G8+ A	CACTCACTGTTAGCGTCATAG	G	152 to 171	
G9+ B	TAGTCCTCAAGCAAGTCCTCG	G	68 to 87	
G1 +A	GGGACAAGTATCTCTATG	G	1 to 18	
G1+ B	GGGACAAGTATCCAGATG	G	1 to 18	
F13- B	CCCGACTTTGTTGCTGCCAA	F	1187 to 1206	Naylor *et al*. (1998)
F11+ B	GTCAGATTAGCCTCATGCTGAAC	F	728 to 750	
F10 –B	AGGCTACCATGCTTACAGGG	F	1116 to 1135	

The nucleotide positions correspond to those in the sequence of type B strain UK/8/94, GenBank Accession number Y14294

The initial denaturation step was done at 94°C for 10 min, followed by 35 cycles (94°C for 1 min, 51°C for 1 min and 72°C for 2 min), a final elongation step at 72°C for 10 min was performed. The size of the amplicons was examined according to the target gene amplified (Tables 1 and 2) and according to the literature (Cavanagh *et al*., 1999). Each reaction was analyzed by 1.5% agar gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet rays using 100 bp DNA ladder as a marker (Qiagen, Germany) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit with automatic sequencer (ABI-3130, Applied Biosystems, Foster City, CA). Nucleotide and amino acid sequence analysis as well as Phylogenetic analysis were carried out using the sequences of PCR products of F and G genes of the Egyptian isolates and vaccine strains. Multiple and pairwise sequence alignments were done by using the ClustalV algorithm of BioEdit software and MegAlign program from LaserGene Biocomputing Software Package (DNASTAR, Madison, WI). The phylogenetic tree of the G gene was constructed using the neighbour-joining method of MEGA6 software (Tamura *et al*., 2013) as well as the evolutionary divergence between sequences was also estimated using the same software. The partial sequences of the F gene and G gene of the Egyptian isolate (Giza-TRT-4-2009) were submitted to Genbank and accepted as HQ677586 and JX647840, respectively.

Results

Results of RT-PCR for aMPV

Eight samples (Giza-TRT-1, Giza-TRT-2, Giza-TRT-3, Giza-TRT-4, Giza-TRT-5, Beni-Suif-TRT-7, Cairo-TRT-8 and Giza-TRT-10) were positive by RT-PCR for direct detection of aMPV and showed two specific bands at 489 bp and 408 bp of F gene in nested RT-PCR (Table 2).

The results indicated that the 2 vaccines used, Aviffa® and Vaccine Nemovac®, were positive for F gene by nested PCR. Both PCRs were positive either by using the 2 primer pairs “F13B+F11B” that yielded a product of 489 bp, or by the nested primer pairs “F10B+F11B” that yielded a product of 408 bp (Table 2). The eight samples (Giza-TRT-1, Giza-TRT-2, Giza-TRT-3, Giza-TRT-4, Giza-TRT-5, Beni-Suif-TRT-7, Cairo-TRT-8 and Giza-TRT-10) were positive by RT-PCR for direct detection of aMPV and produced a specific 444 bp of G gene using 2 sets of primers G1A+G5- and G1B+G5- and by the nested primer pairs G9B+G5- that yielded a product of 361 bp specific for B subtype while they were negative using the nested primer pairs G8A+G5- that specific for A subtype (Table 2).

The results of nested PCR for G gene indicated that the 2 vaccines used, Vaccine Aviffa® and Vaccine Nemovac®, as well as samples (Giza-TRT-1, Giza-TRT-2, Giza-TRT-3, Giza-TRT-4, Giza-TRT-5, Beni-Suif-TRT-7, Cairo-TRT-8 and Giza-TRT-10) were positive for both PCRs using the 2 primer pairs “G1A+G5- and G1B+G5-” that yielded a product of 444 bp and by the nested primer pairs “G9B+G5-” that yielded a product of 361 bp specific for B subtype while both vaccines tested negative using the nested primer pairs G8A+G5- that were specific for A subtype (Table 2). These results indicated that the 2 vaccines as well as those samples were related to B subtype of aMPV. Samples TRT-6 and TRT-9 showed negative PCR results indicating that the virus is not the cause of the signs and there are other causes of respiratory disease in turkeys in these flocks.

Results of Virus Isolation and Propagation

The aMPV isolation and propagation was done in embryo chicken eggs via yolk sac inoculation route and further passed twice in Vero cells (Fig. 1). Virus isolation was completed for five out of the ten samples (Giza-TRT-1, Giza-TRT-3, Giza-TRT-4, Beni-Suif-TRT-7 and Cairo-TRT-8). These five isolates were confirmed positive by RT-PCR for aMPV and produced a specific 489 bp of F gene.

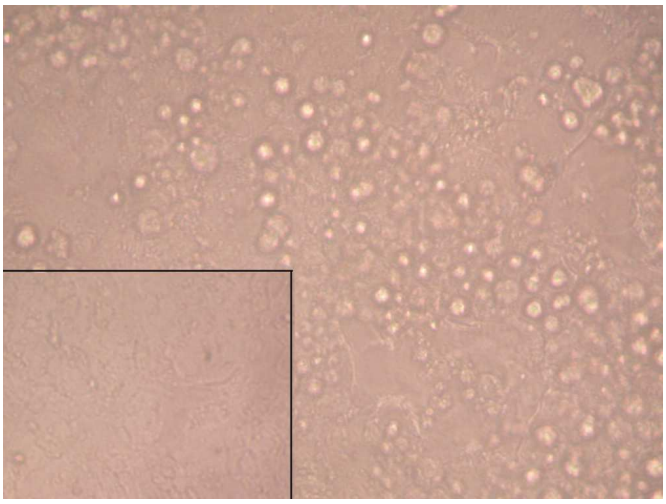


Fig. 1. Vero cells infected with aMPV of isolate Giza-TRT-4 after 72 h post infection, the infected cells showed vacculation, rounding and aggregations in comparison to normal non infected negative control at the lower left corner of the figure

Table 2. Results of RT-PCR for F gene and G gene

	F gene		G gene			
	1st PCR	Nested PCR	1st PCR		Nested PCR	
	F13B+F11B	F10B+F11B	G1A+G5	G1B+G5	G8A+G5	G9B+G5
Sample	(489 bp)	(408 bp)	(444 bp)	(444 bp)	(268 bp)	(361 bp)
Vaccine Affifa®	+	+	+	+	-	+
Vaccine Nemovac®	+	+	+	+	-	+
Giza-TRT-1	+	+	+	+	-	+
Giza-TRT-2	+	+	+	+	-	+
Giza-TRT-3	+	+	+	+	-	+
Giza-TRT-4	+	+	+	+	-	+
Giza-TRT-5	+	+	+	+	-	+
Beni-sueif-TRT-6	-	-	/	/	/	/
Beni-sueif-TRT-7	+	+	+	+	-	+
Cairo-TRT-8	+	+	+	+	-	+
Giza-TRT-9	-	-	/	/	/	/
Giza-TRT-10	+	+	+	+	-	+

The 1st PCR for F gene was done using primers F13B+F11B with positive product of 489 bp, while the Nested PCR was done using primers F10B+F11B with positive product of 408 bp

Results of Sequencing and Genetic Analysis

The amplified part of the F and G genes from the field viruses were sequenced and compared with the other aMPV strains. (Fig. 2-6).

The nucleotide sequence analysis of the F gene revealed minor changes between field isolates and vaccine strains used in this analysis. The most characteristic change was reported in site 102 where it was “T” in vaccine strains while it was “C” in another field isolates, also G220A could differentiate only between Nemovac® and Aviffa® vaccine as well as other field strains but could not differaentiate between Aviffa® and other field isolates (Fig. 2). The only characteristic amino acid change was shown in position 74 (E74K) of F gene that can differentiate Nemovac® than other strains in this analysis but not differentiate between Aviffa® and field isolates (Fig. 3)

The nucleotide percent identity between Egyptian isolate (Giza-TRT-4) and other field and vaccine strains from Europe for the F gene ranged from 98.5% to 98.9%, while it was 99.6% and 99.8% of the 2 vaccines used in the comparison (Nemovac® and Aviffa®), respectively and it was 99.1% with vaccine ancestor strain VCO3 (Fig. 4). These data indicate a close relatedness of Egyptian viruses to vaccine strains.

The nucleotide sequence comparison showed similarity of Egyptian isolates and characteristic single nucleotide change at position 107 (A) in comparison to aMPV (strain 6574) attachment protein (G), while there was another change in position 270 (G to A) in the Egyptian isolates similar to strains 872S and 6574 but different from other strains from Nigeria, Korea, Israel and Brazil (Fig. 5). The mutation at this site caused amino acid change at protein level from Glycine to Agrinine G/R in Egyptian viruses and in strain 672S differentiating them from VCO3 strain and other strains (Fig. 6).

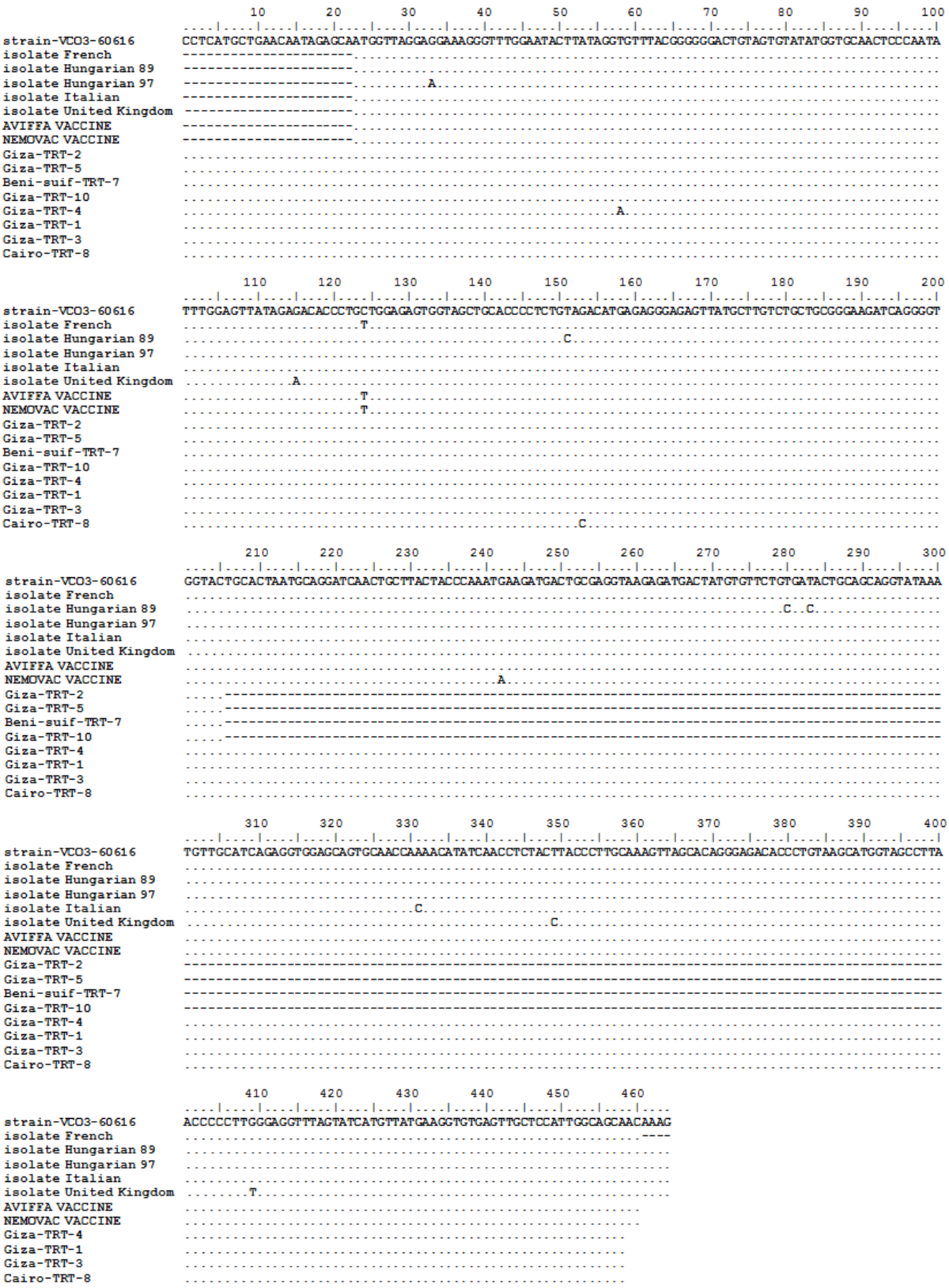


Fig. 2. Partial F gene nucleotides sequence comparison of field viruses of aMPV

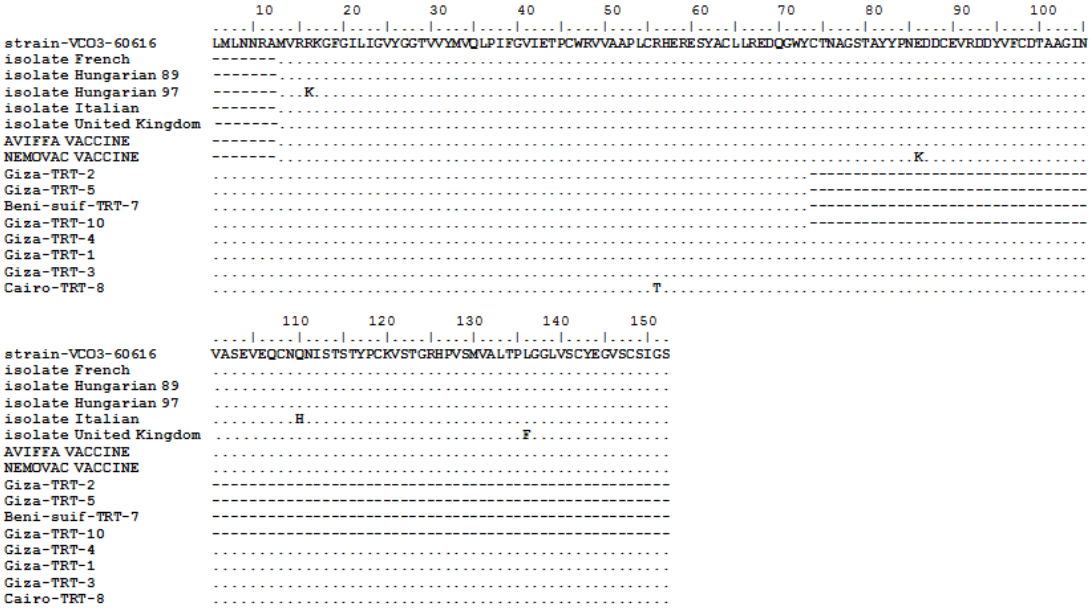


Fig. 3. Partial F gene amino acid sequence comparison of field viruses of aMPV

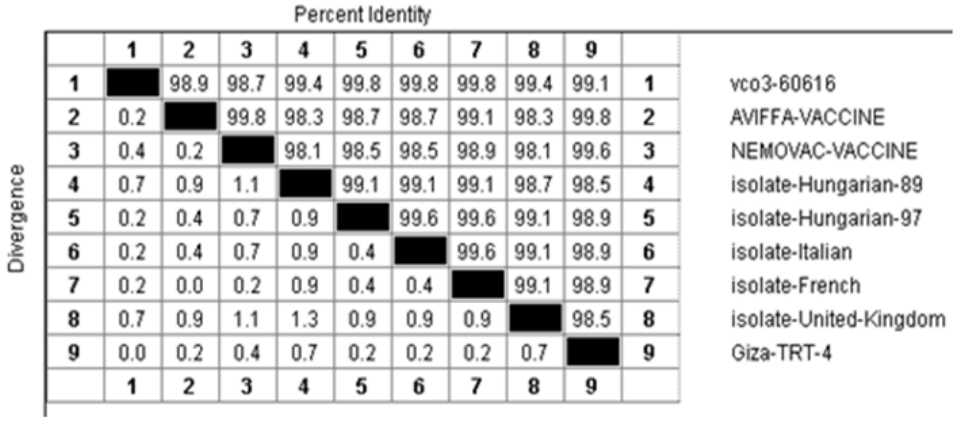
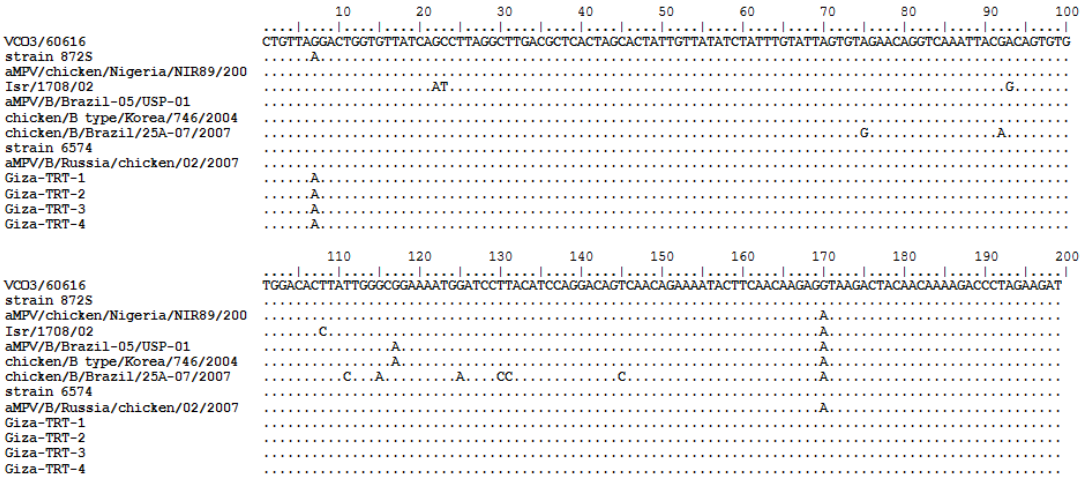


Fig. 4. The percent identity of F gene nucleotides sequence comparison of field viruses of aMPV



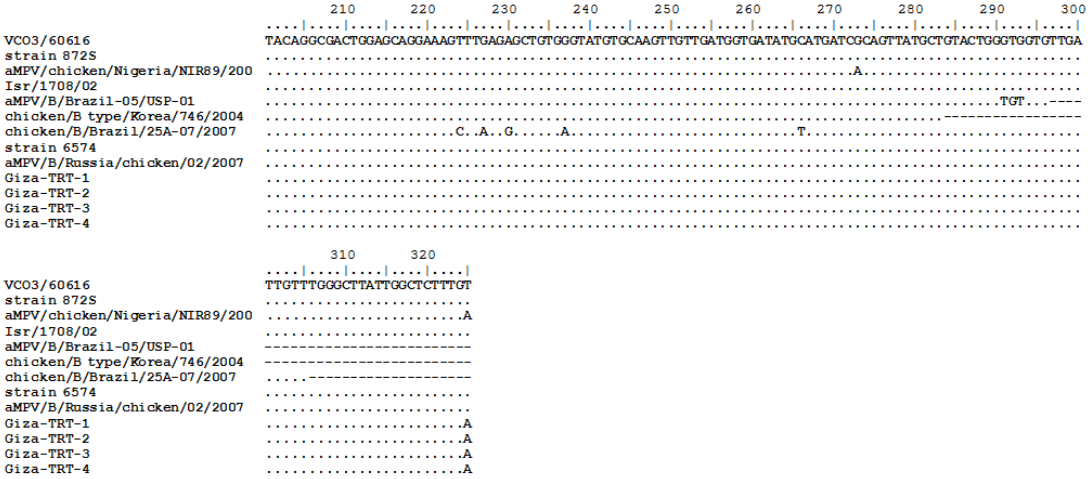


Fig. 5. Partial G gene nucleotide sequence comparison of field viruses of aMPV, the analysis was done based on attachment G gene nucleotide sequences

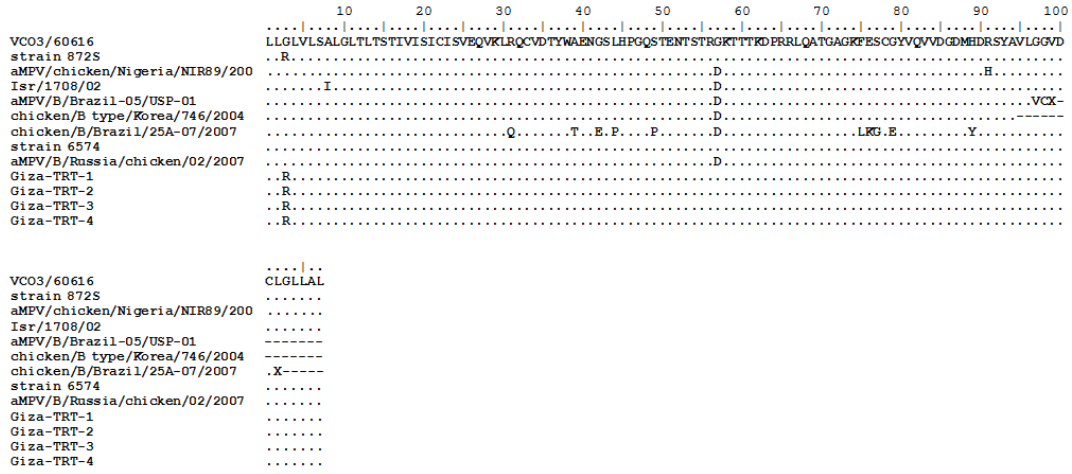


Fig. 6. Partial G gene amino acid sequence comparison of field viruses of aMPV, the analysis was done based on attachment G gene amino acid sequences

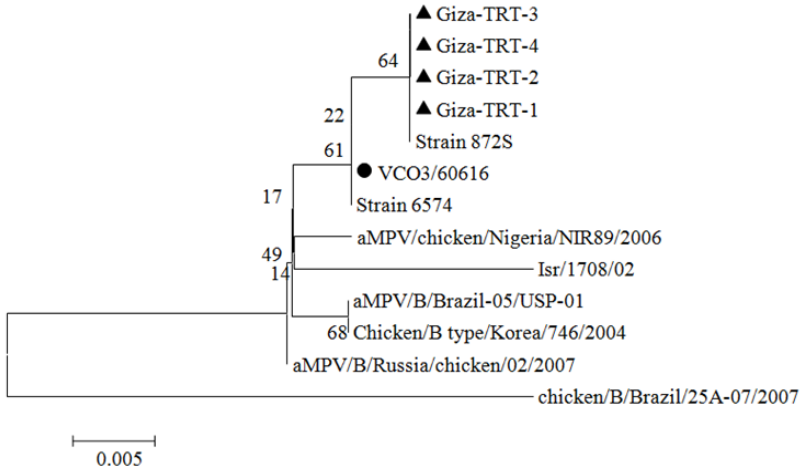


Fig. 7. Phylogenetic analysis of aMPV field isolates, the analysis was done based on fusion F protein

Table 3. Estimates of evolutionary divergence between sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. VCO3/60616													
2. strain 872S	0.00												
3. aMPV/chicken/Nigeria/NIR89/2006	0.01	0.01											
4. Isr/1708/02	0.02	0.02	0.02										
5. aMPV/B/Brazil-05/USP-01	0.01	0.01	0.01	0.02									
6. chicken/B type/Korea/746/2004	0.01	0.01	0.01	0.02	0.00								
7. chicken/B/Brazil/25A-07/2007	0.05	0.06	0.05	0.06	0.05	0.05							
8. strain 6574	0.00	0.00	0.01	0.02	0.01	0.01	0.05						
9. aMPV/B/Russia/chicken/02/2007	0.00	0.01	0.00	0.01	0.00	0.00	0.05	0.00					
10. Giza-TRT-1	0.00	0.00	0.01	0.02	0.01	0.01	0.06	0.00	0.01				
11. Giza-TRT-2	0.00	0.00	0.01	0.02	0.01	0.01	0.06	0.00	0.01	0.00			
12. Giza-TRT-3	0.00	0.00	0.01	0.02	0.01	0.01	0.06	0.00	0.01	0.00	0.00		
13. Giza-TRT-4	0.00	0.00	0.01	0.02	0.01	0.01	0.06	0.00	0.01	0.00	0.00	0.00	

The numbers of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). The analysis involved 13 nucleotide sequences of aMPV G gene. Evolutionary analyses were conducted with MEGA6 (Tamura *et al*., 2013)

The alignment and percent identity of the G gene identified the highest similarity among Egyptian viruses (up to 100%). The highest divergence was shown with chicken/B/Brazil/25A-07/2007 strain (6%), while it was 2% with strain Isr/1708/02. The minimum divergence (0.00) was obtained with strains 872S, 6574 and VCO3 (Table 3).

The phylogenetic tree revealed clustering of Egyptian viruses with strains 872S and 6574 and somewhat differentiated from other related B subtype strains used in comparison of G gene sequencing analysis (Fig. 7).

Discussion

The aMPV causes problems in both turkeys and chickens due to moderate to acute respiratory tract infection with a sudden onset and rapid spread through flocks. Based on genetic and serological properties, aMPV can be classified into four distinct subgroups A, B, C and D (OIE, 2008). The first of B subtype of aMPV was isolated in Europe in 1986 from France. However, the aMPV isolated in the UK in the previous year was A subtype. Subsequently, in 1988 and 1989, aMPV B subtype strains were isolated in Italy, Hungary and Spain. B subtype of aMPV was not detected in the UK until 1994 (Naylor *et al*., 1997). Furthermore, many reports described the detection of A and B subtypes (Cook and Cavanagh, 2002) of aMPV from turkeys and chickens in the Middle East, Asia and Africa.

This work aimed to characterize aMPV from infected turkey flocks in Egypt based on partial sequencing of F and G genes and to differentiate between field and vaccine strains. In this study, aMPV was detected in 8 samples collected from 3 governorates in Egypt (Giza, Beni-Suif and Cairo) from clinically suspected turkey farms. These samples tested positive by RT-PCR for F and G genes. Virus isolation was successful for five isolates through inoculation in chicken eggs and on Vero cells. Further molecular detection using RT-PCR for G and F genes confirmed the five positive isolates of avian metapneumovirus. Analysis of nucleotide sequence relatedness of viral strains of aMPV indicates that the Egyptian viruses were very closely related to each other. The data indicated that these viruses belong to B subtype viruses isolated from Europe. These results raised the concern about vaccination against aMPV and vaccine use in the field. The results suggested that the aMPV was spreading in Egypt over the years due to continuous exposure to aMPV from respiratory illness in the examined farms. However the results are not sufficient to study disease prevalence, but they indicate wide spread nature of infection as the examined farms were distributed in 3 different governorates at middle and middle-south part of Egypt.

The nucleotide sequence comparison for both F and G genes revealed that the Egyptian viruses have highest similarities among each other. The presence of characteristic single nucleotide change in the Egyptian viruses at position 107 (A) in comparison to strain 6574 attachment G protein, while there was another change in position 270 (G to A) similar to strains 872S and 6574 but different from other strains from Nigeria, Korea, Israel and Brazil. This G/A mutation at position 7 of the G gene (Fig. 5) was recently identified as a marker for the AVIFFA/VC03 vaccine (Listorti *et al*., 2014). They showed that an A rather than T at this position was characteristic of the field strain VC03 being converted to its derived VC03 vaccine (Aviffa/Rhinovax).

Another two VC03 vaccine/progenitor marker positions (3210 and 3391) (Listorti *et al*., 2014) were also found indicating a greater certainty that the Egyptian viruses were indeed VC03 vaccine derived.

Some researchers indicated that very few mutations are needed to establish or remove attenuation from vaccine strain (Catelli *et al*., 2006). In another study for

comparison of nucleotide (nt) and predicted amino acid (aa) sequences of parental pathogenic strain VCO3/60616 with the attenuated vaccine strain VCO3/50 revealed higher homology with aMPV A subtype virus (Sugiyama *et al*., 2010), they characterize a total of 18 nt and 10 deduced aa differences between the strains and concluded that one or a combination of several differences could be associated with attenuation of VCO3/50. In this study, the most obvious amino acid changes were noticed in sites G31R and D85G (the numbering from the start codon of the G gene and displayed in Fig. 6 as partial sequencing started from AA29) where the Egyptian isolates shared together with strain VCO3 and 872S in site 31 and with strain 6574 in site 85 and differentiated from other related B subtype stains from Nigeria, Israel, Korea and Brazil (Fig 6).

In previous work, Serine residue in the G proteins and lysine residue in the F protein were found to be exclusive to some field Italian viruses, indicating that those viruses probably originated within Italy and evolved from earlier Italian progenitors (Cecchinato *et al*., 2010). They also indicated that the Italian field viruses evolved in response to selection pressure from vaccine induced immunity.

The phylogenetic tree indicates clustering of the Egyptian field isolates with other European strains with a slight deviation from the vaccine strains indicating that these field isolates may have a common progenitor originated from vaccine strains.

The percent identity between each other and the vaccine strains (Aviffa® and Nemovac®) was very high (more than 99%) indicating high homology between field and vaccine strain used.

Ongor *et al*. (2010) detected 36 positive samples and they all belonged to B subtype by molecular genotyping using primers specific to the attachment glycoprotein (G) gene. Partial sequence analysis of DNA of those samples showed 95% homology between the field type and the reference strain aMPV B subtype. Whereas clinically healthy turkeys were vaccinated with A subtype virus vaccine, the flocks with respiratory problems were vaccinated with B subtype virus vaccine (Ongor *et al*., 2010).

The field reversion to virulence of live vaccines is more likely to occur in avian than in other species because opportunities for horizontal transfer of vaccines have increased a lot due to the higher numbers of vaccination. Some other reports revealed aMPV vaccine reversion to virulence in turkeys, the disease was observed at 3 weeks of age, which was shown to be caused by a vaccine derived reverting virus (Catelli *et al*., 2006) and in that instance, the flocks had been vaccinated at one day of age with the same live A subtype vaccine. At the same time, there was a detailed report about another Italian A subtype aMPV isolated during a turkey rhinotracheitis outbreak on a large farm of seven-week-old male turkeys which had only been vaccinated with B subtype vaccine on the seventh day of birth (Lupini, 2011).

In this study, we characterized these vaccine-derived viruses from different vaccination regimens as well as from non-vaccinated flocks which are very close to other infected flocks indicating circulation of these viruses in the field and they were capable of infecting turkeys and incurring the disease. All vaccination regimens contained at least one live vaccine from B subtype and this supports the idea that these filed viruses were originated from vaccine progenitor.

The sequence analysis of the Egyptian viruses clearly showed they were derived from vaccine of the B subtype applied in the field and indicated evidence of reversion of attenuated vaccine virus to virulence. This explanation was also described by others (Catelli *et al*., 2006) that the vaccine virus is residing in the poults in some region beyond the immediate upper respiratory tract. It may be relevant to note that after some researchers applied an attenuated aMPV which was almost identical to the A subtype vaccine, it could not be detected up to 3 weeks later, turkeys were shown to be fully protected against virulent challenge (Naylor *et al*., 1998). Virus detections were much later at about 2-5 weeks post vaccination. A previous study showed that when the same vaccine was tested for reversion to virulence trials in experimental conditions, disease was seen after 4 to 10 back passages (Naylor and Jones, 1994). In addition, multiple use of vaccines has adverse effects on their efficacy (Ganapathy *et al*., 2006).

Conclusion

The genotyping analysis and phylogenetic analysis based on F and G genes indicate that the Egyptian field viruses are very similar to the European strains of B subtype of aMPV but with slight deviation from the vaccine strains. This indicates that these field isolates may have a common progenitor originated from vaccine strains. This is the first report describing the emergence of vaccine-derived mutants from the B subtype aMPV in broiler turkey farms in Egypt. This work also shed some light on possible reversion of attenuated vaccine viruses to virulence through their passages in the field.

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Author Contributions

The authors contributed to this work as follows:

Abdel-Satar Arafa: He is the corresponding author and he designed, followed up and reviewed all the technical work and he prepared the manuscript.

Wessam Mady: She is the person on charge for laboratory technical work.

Ahmed Hussein, Sabry Tamam and Hanafy Madbouly: They were sharing sample processing, field observations and they approved, reviewed and followed up the work.

Conflicts of Interest

The authors declare no conflict of interest.

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