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The matrix gene of influenza A H5N1 in Egypt, 2006–2016: molecular insights and distribution of amantadine-resistant variants

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Abstract Large-scale sequence analysis of Matrix (M) gene and its coding proteins M1 and M2 was performed for 274 highly pathogenic avian influenza viruses H5N1 circulated in Egypt from 2006 to 2016. The aim is to study the amantadine-resistant markers distribution and to estimate the evolutionary rate. 246 viruses were obtained from the Global Initiative on Sharing All Influenza Data base, and 28 additional viruses were sequenced. Maximum clade credibility (MCC) phylogenetic tree revealed that the M gene has evolved into two different lineages. Estimated Evolutionary analysis showed that the M2 protein possessed higher evolutionary rates (3.45×10^{-3}) than the M1 protein (2.73×10^{-3}). M gene encoding proteins revealed significant markers described to be associated with host tropism and increase in virulence: V15I, N30D, and T121A in M1 and L55F in M2 protein. Site analysis focusing attention on the temporal and host distribution of the amantadine-resistant markers was carried out and showed that vast majority of the M2 amantadine-resistant variants of clade 2.2.1.1 ($n = 90$) is N31 marker, in addition to G27 ($n = 7$), A27 ($n = 5$), I27 ($n = 1$), and S30 ($n = 1$). In 2010–2011, amantadine resistant frequency increased considerably resembling more than half of the resistant variants. Notably, all viruses of clade 2.2.1.1 possessed amantadine-resistant marker. However, almost all current circulating viruses in Egypt of clade 2.2.1.2 from 2014 to 2016 did not carry any amantadine-resistant markers.

Keywords Avian influenza H5N1 · Matrix gene · M1 protein · M2 protein · Amantadine resistance

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Introduction

Highly pathogenic avian influenza (HPAI) H5N1 remains one of the serious threats to poultry worldwide. Numbers of reported cases in different countries have been reported with zoonotic evidence [1]. Since its introduction in Egypt, in 2006, highly pathogenic avian influenza H5N1 (HPAI) virus has been widely spread in a short time among poultry in commercial sectors and backyards, which leads to devastating economic losses each year [2], and continues to be isolated from different poultry sectors from different governorates [3]. Moreover, current human infections by HPAI H5N1 in Egypt still take place; in 2014–2015, unprecedented peak in human cases was observed, and four further cases were reported (till March 2016) [4]. The phylogenetic evolutionary analysis of these viruses indicated that two major H5N1 clades are co-circulating in poultry and human in Egypt; clade 2.2.1.1 and 2.2.1.2 [5–7]. Recent genetic studies showed that viruses of 2.2.1.2 clade became dominant over clade 2.2.1.1 [8, 9].

Influenza A virus segment 7, M gene, encodes two proteins, a highly conserved 252-amino acid M1 and

97-amino acids membrane protein M2 [10]. M1 protein bears the nuclear localization sequence (NLS) motif 101-RKLKR-105 important for membrane binding, self-polymerisation, and nuclear export of ribonucleoproteins (RNPs) [11, 12]. The M2 protein plays a role in genome packaging and formation of virus particles [13]. The transmembrane domain of M2 has ion channel activity, which is the target of amantadine antiviral drugs [14, 15]. Five amino acid sites in the transmembrane region of the M2 protein, L26, V27, A30, S31, and G34, involve in resistance to the antiviral drug amantadine [16–18]. The majority of resistant influenza A viruses (95 %) possess S31N marker [19]. In Egypt, amantadine resistance was associated mainly with S31N from commercial poultry farms [20]. The role of the application of prohibited amantadine as a cheap drug against influenza viruses on the appearance of such mutations is still under study [21].

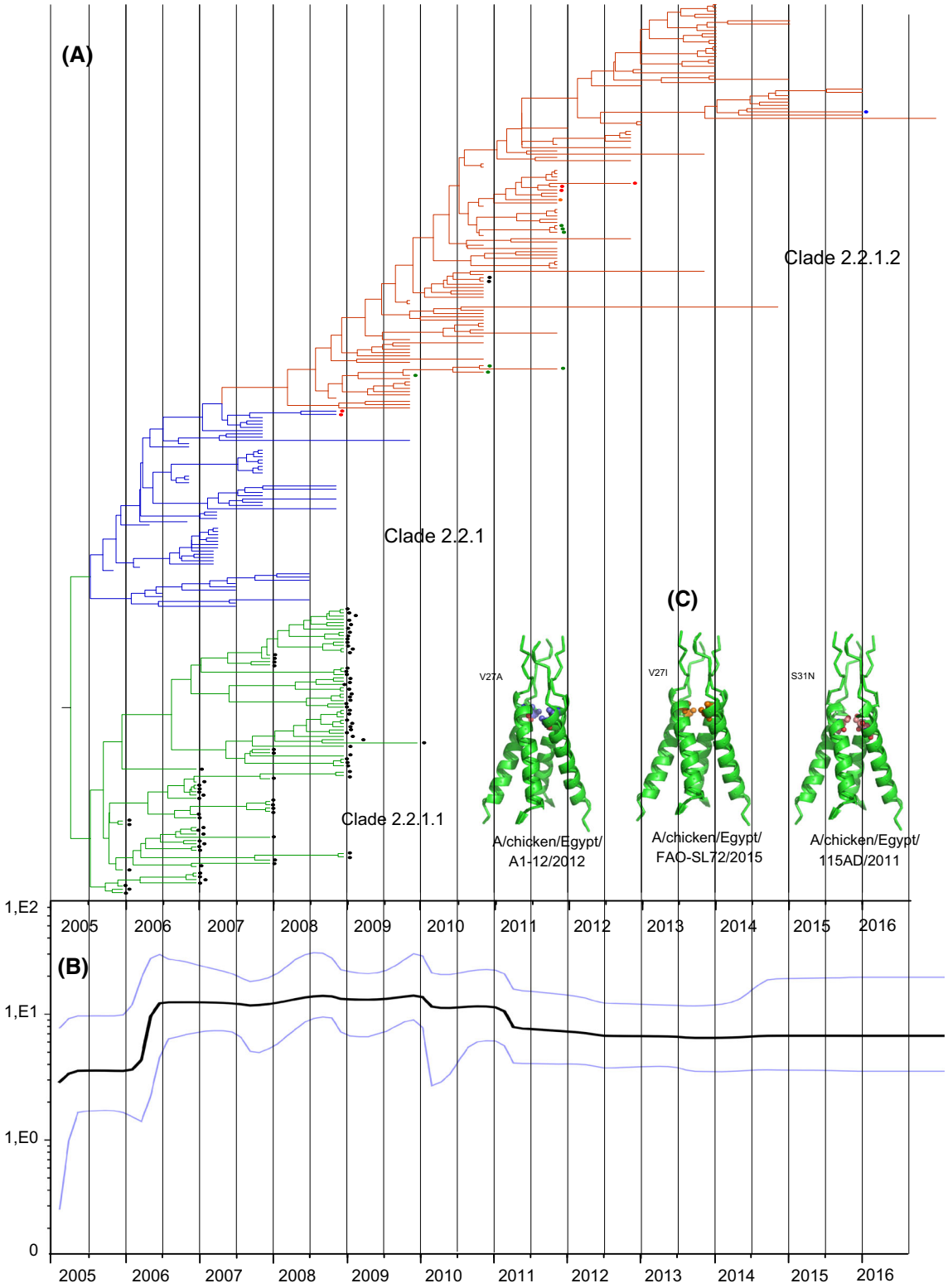
In the current study, twenty-eight highly pathogenic avian influenza H5N1 viruses, recovered from tracheal swabs of diseased chickens, were collected in the period between 2006 and 2016 in 18 governorates from backyard, commercial farm sectors, and live bird market (LBM). Samples were submitted to the National Laboratory of veterinary quality control on poultry production (NLQP) as a part of national surveillance for further identification and isolation. More details about age, governorates, and type of breeding sector are provided in Table S1. Sequences of the M gene of the 28 viruses were obtained, compiled, and analyzed. Generated sequences were submitted to the GenBank database (table S1). Further, genetic sequences of the M gene of 248 H5N1 viruses from avian and human host in Egypt were retrieved from the Global Initiative on Sharing All Influenza Data base GISAID (total = 276) compromising available sequences on GenBank and Influenza Research Database, where partial sequences (<900 bp) were excluded. Maximum clade credibility (MCC) phylogenetic tree was estimated based on Markov Chain Monte Carlo (MCMC) method implemented in the Bayesian Evolutionary Analysis Sampling Trees (BEAST) program version 1.8 [22] after identification of the best-fit model using jModelTest 2.1.7 [23]. The tree was analyzed by the Tree Annotator program included in the Beast package after a 10 % burn-in. Tree was finally viewed and edited using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/). The tree representing the HA defined clades; clades 2.2.1 and its descendant's clade 2.2.1.1 and 2.2.1.2.

Rates of nucleotide substitutions per site per year (subs/site/year) and the 95 % highest posterior density (HPD) were estimated for each protein of the M gene, by using strict molecular clock models. The MCMC computation was done under a Bayesian skyline coalescent tree prior using simulations for 100×10^6 generations with sampling every 1000 steps. Only ESS's of >250 was accepted. Moreover, a Bayesian Skyline plot, implemented in BEAST, was used to infer the population dynamics in the M gene segment of Egyptian H5N1 viruses under the base of relative genetic diversity [Net] through time, in which *t* represents the generation time and *e* effective population [24].

Fig. 1 **a** Temporally structured maximum clade credibility phylogenetic tree and Bayesian Skyline plot of the HA gene showing changes in genetic diversity in the Matrix gene of Egyptian H5N1 viruses (2006–2016). Amantadine-resistant markers are depicted in *dots* (*black* N31; *blue* I27; *red* A27; *green* G27; and *orange* S30) **b** Bayesian skyline plot of changing levels of genetic diversity through time for the M gene (276 sequences). A measure of genetic diversity (Net) is given on the *y* axis with 95 % HPD values. **c** Three-dimensional proton channel matrix 2 (M2) tetrameric structure of the influenza depicted different amantadine-resistant markers recorded in this study. The *ribbons* representations were generated using Swiss-Modell online software and edited with the CCP4MG program. The positions of the amino acids A27, I27, and N31 were *highlighted* (Color figure online)

The genetic diversity of the Matrix genes revealed an increase in the evolutionary rate in the period between late 2006 and 2011 (Fig. 1b). The evolutionary rates estimated for the M gene of the Egyptian H5N1 viruses (All = 274, poultry = 210 and human = 64) were 2.87×10^{-3}, 2.55×10^{-3}, and 2.99×10^{-3} substitutions/site/year, respectively (95 % highest posterior density, HPD, 2.36–3.39, 2.0–3.0, and 2.26–3.76). Furthermore, the estimated evolutionary rates were calculated for both M1 and M2 protein coding segment separately. Remarkably, the rates of the two proteins were different: a mean rate of 2.73×10^{-3}, 3.19×10^{-3}, and 2.86×10^{-3} (95 % HPD; 2.20–3.27, 2.51–3.88, and 2.03–3.18) for M1, and a mean rate of 3.45×10^{-3}, 3.26×10^{-3}, and 4.84×10^{-3} (95 % HPD; 2.57–4.34, 2.34–4.17, and 3.03–6.79) for M2., Faster evolution of M2 was observed especially for viruses isolated from humans, in contrast, viruses isolated from poultry showed higher evolutionary rates among their M1-coded protein sequence. The selective pressures (mean dN/dS) estimated by software suite Datamonkey [25] were 0.08 for M1 versus 0.61 for M2 indicating higher selective pressure of the M2 and probably indicates that it is under positive selection pressure for amino acid substitution (*P* value <0.05). The relatively higher evolutionary rates of M2 in comparison to M1 along with positive selection pressure may be due the misuse of amantadine in poultry feeds as antiviral to reduce the high losses of birds and mortalities due to H5N1 infections in specific time point related to the emergence of clade 2.2.1.1 in Egypt in late 2007.

M1 protein showed substitution mutations associated with increased virulence in mammals (markers V15I and N30D) in all isolates [26]. Further, T121A mammalian-


(A)
Clade 2.2.1.2
Clade 2.2.1
Clade 2.2.1.1
(C)
V27A
V27I
S31N
A/chicken/Egypt/
A1-12/2012
A/chicken/Egypt/
FAO-SL72/2015
A/chicken/Egypt/
115AD/2011
(B)
1,E2
1,E1
1,E0
0
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016

adaptation mutation was observed in one isolate A/chicken/Egypt/A1-2012/2012, and this mutation was reported in only one virus isolated from human case in 2011 (A/Egypt/N7724/2011) and was previously seen in 1918 influenza pandemic virus [27]. In the M2 protein, new mutation among the Egyptian H5N1 viruses was recorded at position 55 (L55F) in A/chicken/Egypt/S38/2014 virus of the new cluster of viruses of clade 2.2.1.2 which emerged in 2014–2015 [5]. The later mutation was described to be associated with enhanced transmission to human [28] (Table S2). M1 contains the nuclear localization sequence (NLS) motif 101-RKLKR-105 at its N-terminal domain (residues 1–164) [11, 29], and R101K substitution mutation was observed in all viruses sequenced in this study. Also, the obtained data showed the presence of R95K in the matrix gene of 20 viruses, which was described to have a critical role on the filamentous morphology of virus particles [30].

It was accepted that the acquisition of amantadine-resistant markers and the emergence of amantadine-resistant viruses were related to the effect of drug pressure [31, 32]. In Egypt, although the usage amantadine is prohibited, amantadine products were found to be used in poultry farms [33]. 37.9 % of the analyzed Egyptian H5N1 influenza A viruses revealed individual amantadine-resistant markers. Residues conferring resistance to amantadine were identified including G27 ($n = 7$, 2.5 %), A27 ($n = 5$, 1.8 %), I27 ($n = 1$, 0.4 %), S30 ($n = 1$, 0.4 %), and N31 ($n = 90$, 32.8 %) in the analyzed 274 viruses where three different markers were observed in the viruses sequenced in this study (Table 1). All amantadine-resistant markers were observed as individual cases, and no multiple sites of resistance in the same strain were recognized. Notably, the S31N resistance variants of clade 2.2.1.1 showed a gradual increase starting from late 2006 until reaching the peak with 58 cases in 2010 followed by sharp decrease in 2011. The increase in the number of amantadine-resistant marker in this period is assumed to be associated with the start of illegal usage of amantadine in poultry sectors [33] and in association with the emergence of the viruses of clade 2.2.1.1, and this evidence is correlated with the increased evolutionary rate observed during this period (Fig. 1b). Amantadine-resistant marker was observed in one virus (A/Egypt/N11981/2009) which was reported previously as oseltamivir-resistant virus [20], indicating the dual resistance of this virus against both antiviral drugs. Moreover, amantadine-resistant marker V27I was observed for the first time in the Egyptian viruses in one recent virus isolated in late 2015. This marker was also observed in viruses isolated from West Africa in the same year (Nigeria and Ghana), and they were belonging to clade 2.3.2.1c. Viruses harboring amantadine-resistant markers are shown in bold face in table S1. The position of the resistance markers are shown on the 3D protein structure of the M2 protein of three representative viruses obtained in the frame of this study (Fig. 1c) generated using Swiss-Model prediction server [34] and viewed by CCP4MG software version 2.10.5 [35]. Amantadine-resistant viruses were identified in different hosts including human and avian hosts; this raises a problem during antiviral drugs application for disease treatment in human. Further, in 2014, amantadine became prohibited in the treatment of human influenza A infections due to its inefficacy [36]. There are no limitations based on the type of breeding sectors or the geographical distribution (Table S1). It was noticed from the phylogenetic analysis performed in this study that all viruses of clade 2.2.1.1 were associated with amantadine-resistant marker. It is not clear whether the amantadine-resistant viruses were

Table 1 The temporal and host distribution of the Amantadine-resistant markers in Egyptian H5N1 viruses

	L26	V27I	V27G	V27A	A30S	S31N	G34	Total
Host								
Chicken	–	1	3	1	–	84	–	89 (32.4 %)
Duck	–	–	–	–	–	4	–	4 (1.4 %)
Turkey	–	–	–	–	–	1	–	1 (0.4 %)
Human	–	–	4	4	1	1	–	10 (2.7 %)
Total	–	1 (0.4 %)[a]	7 (2.5 %)	5 (1.8 %)	1 (0.4 %)	90 (32.8 %)		104 (37.9 %)
Year								
2006–2007	–	–	–	–	–	6	–	6 (2.2 %)
2008–2009	–	–	1	2		35	–	38 (13.9 %)
2010–2011	–	–	6	2	1	49	–	58 (21.2 %)
2012–2013	–	–	–	1	–	–	–	1 (0.4 %)
2014–2016	–	1		–			–	1 (0.4 %)
Total	–	1 (0.4 %)[a]	7 (2.5 %)	5 (1.8 %)	1 (0.4 %)	90 (32.8 %)	–	104 (37.9 %)

A total of 274 viruses were involved in this analysis. There were only 104 viruses which carried Amantadine-resistant marker(s), while the remaining was not

[a] Percent from the total number of viruses included in the study ($n = 274$)

emerged randomly or due to the administration of amantadine to poultry over all the years or only at a few times, and these mutations became fixed in the virus population and maintained without continuing selection pressure. Moreover, the role of amantadine administration or its stop in the almost extinction of viruses of clade 2.2.1.1 is also not clear and still needs to be investigated. Notably, the major amantadine-resistant marker, S31, is observed in only one human virus.

Nevertheless, given the importance of amantadine in the prevention and treatment of influenza in humans, it is crucial to raise concerns about the sporadic isolation of amantadine-resistant variants in Egypt and highlight the importance of monitoring and controlling the improper use of antivirals.

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Compliance with ethical standards

Samples collection by veterinarians was achieved from poultry sectors in Egypt. Obtaining swab samples from the trachea of poultry is minimally invasive and does not require any anesthesia for the animal; minimal restrainment is used for a very short time.

Conflict of interest All the authors declare that they have no conflict of interest.

References

1. S. Briand, J.S. Tam, M. Bloch, E. Mumford, Procedia Vaccinol. **2**, 159–165 (2010)
2. E.M. Abdelwhab, H.M. Hafez, Epidemiol. Infect. **139**, 647–657 (2011)
3. E.M. Abdelwhab, M.K. Hassan, A.S. Abdel-Moneim, M.M. Naguib, A. Mostafa, I.T. Hussein, A. Arafa, A.M. Erfan, W.H. Kilany, M.G. Agour, Z. El-Kanawati, H.A. Hussein, A.A. Selim, S. Kholousy, H. El-Naggar, E.F. El-Zoghby, A. Samy, M. Iqbal, A. Eid, E.M. Ibraheem, S. Pleschka, J. Veits, S.A. Nasef, M. Beer, T.C. Mettenleiter, C. Grund, M.M. Ali, T.C. Harder, H.M. Hafez, Infect. Genet. Evol. **40**, 80–90 (2016)
4. WHO, Influenza at the human-animal interface (2016), http://www.who.int/influenza/human_animal_interface/Influenza_Summary_IRA_HA_interface_04_04_2016.pdf?ua=1. Accessed 17 April 2016
5. A.S. Arafa, M.M. Naguib, C. Luttermann, A.A. Selim, W.H. Kilany, N. Hagag, A. Samy, A. Abdelhalim, M.K. Hassan, E.M. Abdelwhab, Y. Makonnen, G. Dauphin, J. Lubroth, T.C. Mettenleiter, M. Beer, C. Grund, T.C. Harder, Euro. Surveill. **20**, 2–8 (2015)
6. M.A. Rohaim, R.F. El-Naggar, M.M. Hamoud, S.A. Nasr, E. Ismael, S.E. Laban, H.A. Ahmed, M. Munir, Transboundary and emerging diseases (2016). doi:10.1111/tbed.12472
7. G.J. Smith, R.O. Donis, World Health Organization/World Organisation for Animal H.F., and Agriculture Organization H.E.W.G. Influenza Other Respir. Viruses **9**, 271–276 (2015)
8. M.M. Naguib, E.M. Abdelwhab, T.C. Harder, Arch. Virol. **161**, 1963–1967 (2016)
9. R. El-Shesheny, A. Kandeil, O. Bagato, A.M. Maatouq, Y. Moatasim, A. Rubrum, M.S. Song, R.J. Webby, M.A. Ali, G. Kayali, J. Gen. Virol. **95**, 1444–1463 (2014)
10. R.A. Lamb, C.J. Lai, P.W. Choppin, Proc. Natl. Acad. Sci. USA **78**, 4170–4174 (1981)
11. Z. Ye, D. Robinson, R.R. Wagner, J. Virol. **69**, 1964–1970 (1995)
12. M.K. Safo, F.N. Musayev, P.D. Mosier, Q. Zhou, H. Xie, U.R. Desai, PLoS One **9**, e109510 (2014)
13. M.F. McCown, A. Pekosz, J. Virol. **79**, 3595–3605 (2005)
14. A. Salter, B. Ni Laoi, B. Crowley, Intervirology **54**, 305–315 (2011)
15. R.B. Belshe, M.H. Smith, C.B. Hall, R. Betts, A.J. Hay, J. Virol. **62**, 1508–1512 (1988)
16. D. Li, R. Saito, Y. Suzuki, I. Sato, H. Zaraket, C. Dapat, I.M. Caperig-Dapat, H. Suzuki, J. Clin. Microbiol. **47**, 466–468 (2009)
17. E.A. Govorkova, T. Baranovich, P. Seiler, J. Armstrong, A. Burnham, Y. Guan, M. Peiris, R.J. Webby, R.G. Webster, Antiviral Res. **98**, 297–304 (2013)
18. A.J. Hay, M.C. Zambon, A.J. Wolstenholme, J.J. Skehel, M.H. Smith, J. Antimicrob. Chemother. **18**(Suppl B), 19–29 (1986)
19. G. Dong, C. Peng, J. Luo, C. Wang, L. Han, B. Wu, G. Ji, H. He, PLoS One **10**, e0119115 (2015)
20. G. Kayali, R.J. Webby, M.F. Ducatez, R.A. El Shesheny, A.M. Kandeil, E.A. Govorkova, A. Mostafa, M.A. Ali, PLoS One **6**, e17730 (2011)
21. E.M. Abdelwhab, J. Veits, T.C. Mettenleiter, Br. J. Virol. **1**, 29–35 (2014)
22. A.J. Drummond, M.A. Suchard, D. Xie, A. Rambaut, Mol. Biol. Evol. **29**, 1969–1973 (2012)
23. D. Darriba, G.L. Taboada, R. Doallo, D. Posada, Nat. Methods **9**, 772 (2012)
24. A.J. Drummond, A. Rambaut, B. Shapiro, O.G. Pybus, Mol. Biol. Evol. **22**, 1185–1192 (2005)
25. A.F. Poon, S.D. Frost, S.L. Pond, Methods Mol. Biol. **537**, 163–183 (2009)
26. R.P. Kamal, J.M. Katz, I.A. York, Curr. Top. Microbiol. Immunol. **385**, 243–274 (2014)
27. D.B. Finkelstein, S. Mukatira, P.K. Mehta, J.C. Obenauer, X. Su, R.G. Webster, C.W. Naeve, J. Virol. **81**, 10292–10299 (2007)
28. M. Shaw, L. Cooper, X. Xu, W. Thompson, S. Krauss, Y. Guan, N. Zhou, A. Klimov, N. Cox, R. Webster, W. Lim, K. Shortridge, K. Subbarao, J. Med. Virol. **66**, 107–114 (2002)
29. S. Arzt, I. Petit, W.P. Burmeister, R.W. Ruigrok, F. Baudin, Virus Res. **99**, 115–119 (2004)
30. S.V. Bourmakina, A. Garcia-Sastre, J. Gen. Virol. **84**, 517–527 (2003)
31. H. Masuda, H. Suzuki, H. Oshitani, R. Saito, S. Kawasaki, M. Nishikawa, H. Satoh, Microbiol. Immunol. **44**, 833–839 (2000)
32. S.A. Harper, K. Fukuda, T.M. Uyeki, N.J. Cox, C.B. Bridges, Advisory committee on immunization practices C.f.D.C., and prevention. MMWR Recomm Rep **54**, 1–40 (2005)
33. I.M. Hussein, E.M. Abdelwhab, Nature middle east (2016)
34. M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. Gallo Cassarino, M. Bertoni, L. Bordoli, T. Schwede, Nucleic Acids Res **42**, W252–W258 (2014)
35. S. McNicholas, E. Potterton, K.S. Wilson, M.E. Noble, Acta Crystallogr. D Biol. Crystallogr. **67**, 386–394 (2011)
36. Reactions weekly 1533, 6–6 (2015)