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Phylogenetic and antigenic analysis of influenza A(H3N2) viruses isolated from conscripts receiving influenza vaccine prior to the epidemic season of 1998/9

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SUMMARY

Roughly half (54%) of the 910 young conscripts at a garrison in Finland were vaccinated with commercial influenza vaccines in autumn 1998. During the influenza outbreak in February 1999, 12 H3N2-subtype virus strains were isolated from vaccinated patients, and 11 such strains were isolated from unvaccinated patients. The isolates were related to the vaccine strain A/Sydney/5/97 and could be classified into three subgroups based on sequence variation in the HA1 gene coding for the variable domain of viral haemagglutinin (HA). A total of 6–10 amino-acid substitutions in HA1, three of these in the receptor-binding site, differentiated the field strains from the vaccine virus. In haemagglutination inhibition (HI) tests, eight strains from the study population exhibited reduced reactivity with a variety of antisera including human post-vaccination sera. Six of these strains were isolated from vaccinated and two from unvaccinated patients. The reduced reactivity did not correlate with particular amino-acid changes in HA1. We suggest that low-reactivity viruses may have an advantage over other co-circulating variants under some circumstances characterized by enhanced immunity-mediated selection and high infection pressure. Whether the frequency of these viruses increased in our vaccinated study population cannot be determined, nor can their effect on vaccine efficacy.

INTRODUCTION

Influenza virus infections are not uncommon among people who have been vaccinated against influenza prior to the epidemic season [1]. Incomplete protection efficacy is more probable when there is a poorer antigenic and genetic match between the vaccine virus and the epidemic viruses. Under circumstances of good antigenic matching, failure of protection may be attributed to a failure of the vaccination to provoke antibodies, or to infection with co-circulating minority virus variants capable of breaking through the vaccine-induced immunity. Although considerable sequence homology within an epidemic usually exists [2], separate lineages and closely related variants can co-circulate [3], and their numbers increase due to imported cases often stemming from international air traffic [4]. During the 1997/8 outbreak of H3N2-subtype influenza A viruses in Finland, virus strains from vaccinated and unvaccinated patients were isolated [5]. Some intra-epidemic genetic and antigenic variability was detected but could not be shown to have significance for the ability of the virus to break through immunity. This was predictable, for in that particular winter the match between the vaccine strain (A/Nanchang/933/95) and the epidemic virus (A/Sydney/5/97-like strains) was not as good as in most years. In the present study we analyse the genetic and antigenic variation of the H3N2-subtype viruses that we isolated from vaccinated and unvaccinated patients in a semi-closed community of young military con-

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scripts during the 1998/9 epidemic season, when the antigenic match between vaccine and circulating strains was closer.

METHODS

Study population and vaccinations

An influenza vaccine was accepted by 54% (494/910) of male conscripts (born 1978–80 apart from a few exceptions) in the Light Infantry Brigade garrisoned at Sodankylä in northern Finland. In all, 203 of the conscripts received the vaccine in the garrison during autumn 1998, while 291 conscripts who entered the service in January 1999 were vaccinated in December 1998 at their place of residence.

Parenteral trivalent influenza vaccines, having WHO-recommended compositions [6], were used. Influvac® (Solvay Pharmaceuticals BV) was used as the vaccine administered in the garrison. The other four vaccines available in the conscripts' places of residence were Begrivac® (Chiron Behring GmbH & Co.), Fluarix® (SmithKline Beecham), Flupar-Vaccin® (Orion) and Vaxigrip® (Pasteur Mérieux Serums et Vaccins). Reassortant virus strain IVR-108, derived from A/Sydney/5/97 (SDN/5/97), served as the H3N2 component in all these vaccines.

Clinical samples and virus isolation

Nasopharyngeal aspirates (NPA) from patients with febrile (38 °C) upper respiratory infection were taken as described previously [5]. Collection was initiated during the first week of February when the first cases of clinical influenza were recorded in the garrison. It was continued for 6 weeks and through two epidemic waves, of which the first was mainly caused by influenza A viruses. Patients who had been vaccinated against influenza were over-represented in the collection to assure a representative number of influenza virus strains capable of breaking through vaccine-induced immunity.

The NPA specimens ($n = 41$) were tested by time-resolved fluoroimmunoassay (TR-FIA) [7] for influenza A and B viral antigens. In the antigen-positive cases, the pretreated (dithiothreitol, final concentration 3–5 mmol/sample) NPAs were inoculated onto Madin–Darby canine kidney (MDCK) cell cultures and cultivated as described previously [5]. A total of 23 influenza A virus strains were isolated from the 29 specimens positive for influenza A when tested by TR-FIA, i.e. 12 strains from vaccinated and 11 strains from unvaccinated patients.

Antigenic and genetic analysis

Preliminary subtyping of the 68 influenza virus strains we isolated in Finland during 1998/9 and more complete antigenic analyses of the 23 strains isolated in the study population (included in the 68 strains) were performed using haemagglutination inhibition (HI) assay as described earlier [8]. However, due to poor reactivity with chicken erythrocytes [9], goose erythrocytes were used instead. Seven antiserum pools were used in the antigenic analyses performed in triplicate, and geometric mean titres (GMT) were calculated. One of the pools consisted of 13 human sera drawn from conscripts in the study population immediately prior to the influenza vaccination in autumn 1998. Another similar pool consisted of 11 sera taken 5 weeks post-vaccination. The remaining five pools consisted of post-immunization rat antisera [8] raised against the virus strains listed in Figure 3. In addition to antigenic analysis of the 23 strains from the study population, 39 strains isolated elsewhere in Finland during the same epidemic season were examined once. In these latter tests, two further replicates were performed with a smaller number of antisera, if the reactivity pattern of the first HI test was ambiguous.

The 23 field strains of the study population, complemented with five strains representing viruses isolated elsewhere in Finland during the same epidemic season and one strain isolated previously (FN/247/92), were analysed for this study by nucleotide sequencing of HA1 (accession nos. AF442455–AF442483; for strain designations, see Fig. 1). RNA extraction, cDNA synthesis, PCR amplification procedures, and sequencing were performed with some modifications [5], as previously described [10]. Evolutionary trees were generated using the DNABOOT and DNAML computer programs of the Phylogeny Inference Package 3.4 (Phylip), which employ the parsimony and maximum-likelihood methods, respectively. The DNABOOT program was also used to calculate bootstrap reliability measures for deduced tree topologies. A total of 500 bootstrap replicates were performed. RasWin Molecular Graphics Windows 2.6 (Copyright © 1993–5 by R. Sayle) was used for locating amino-acid residues on the HA1 model of influenza A/Aichi/68(H3N2) (PDM code 1HGG). A total of 10 strains of the phylogenetic tree

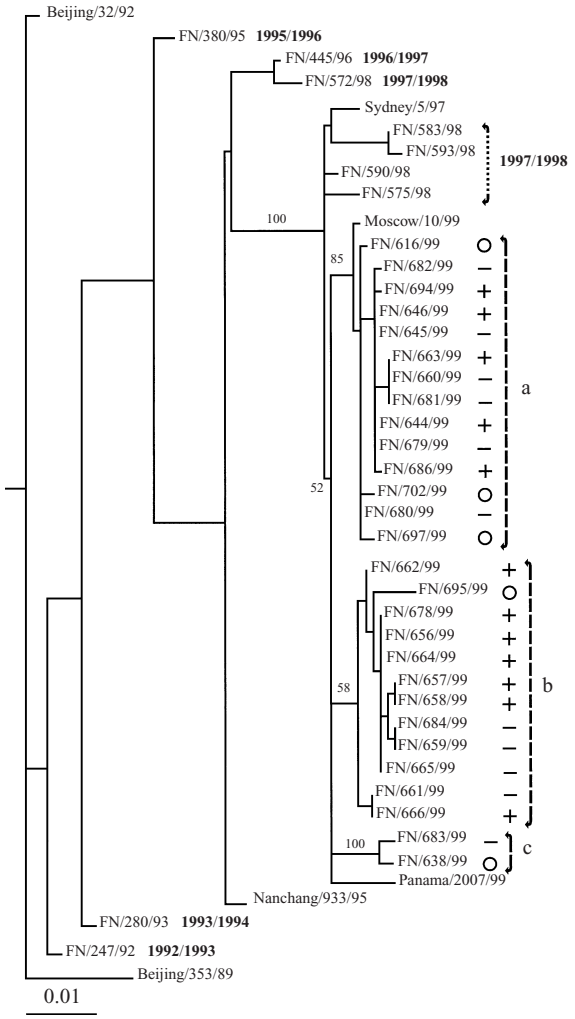

Fig. 1. Phylogenetic relationships of the HA1 domain of the field strains isolated during the 1998/9 epidemic season in the Sodankylä study population, representative field strains from the previous epidemic seasons and some reference strains. The maximum-likelihood method (Phylip, DNAML) was applied. The tree grows to the right, horizontal lines are proportional to the number of nucleotide changes and vertical lines are used for spacing only. The three subgroups that circulated in Finland during 1998/9 are referred to as a, b and c. Similar tree topology was obtained when a maximum parsimony algorithm (DNABOOT) was used; bootstrapping confidence values are indicated. Vaccination status is indicated by + (vaccinated) or − (unvaccinated) when strains from the study population are concerned, while strains isolated elsewhere in Finland during 1998/9 are indicated with an open circle.

shown in Figure 1 were sequenced previously in our laboratory (L76035–Beijing/32/92, L76036–Beijing/353/89, L75981–FN/247/92, AF311677–FN/380/95, AF311678–FN/445/96, AF311680–FN/572/98, AF311683–FN/575/98, AF311689–FN/583/98, AF311695–FN/590/98, AF311697–FN/593/98), two sequences were obtained from GenBank (AF008725–Nanchang/933/95, AF180584–Sydney/5/97) and two sequences (Moscow/10/99 and Panama/2007/99) were obtained from the National Institute for Medical Research, Mill Hill, London.

RESULTS

Phylogeny and amino-acid variability

The 23 virus strains isolated from conscripts in the study population all belonged to the lineage of SDN/5/97-like viruses, which had diverged from the Nanchang/933/95-like viruses of the previous epidemic seasons (Fig. 1). Three subgroups were recorded (a–c), which were separate from the SDN/5/97-like viruses prevalent during the 1997/8 epidemic season in Finland [5]. One of the 1998/9 subgroups was represented by a single field strain (FN/683/99), whereas the remaining two subgroups (a and b) both consisted of 11 field strains. Moscow/10/99, the H3N2-subtype reference strain in the recommended composition of influenza virus vaccines for use during the 2000/1 season [11], belonged to subgroup a. Each of the subgroups was detected in the Light Infantry Brigade within 9 days from the initiation of sample collection, suggesting that they reached the garrison at practically the same time. Viruses isolated from the vaccinated (12 strains) and unvaccinated (11 strains) garrison patients were equally represented in the two major subgroups (Fig. 1). Of the 5 strains isolated outside the study population during 1998/9 in 5 different localities scattered over Finland, 3 strains belonged to subgroup a, 1 to subgroup b and 1 to subgroup c.

A maximum of ten amino-acid substitutions differentiated the MDCK-grown field strains isolated in the study population in 1998/9 from each other, and 6–10 substitutions differentiated these strains from the egg-grown vaccine virus SDN/5/97 (Fig. 2). Only one amino-acid change differentiated all of the 1998/9 strains from the SDN/5/97-like strains that circulated in Finland during the 1997/8 epidemic season [5]: the change from Y to S at position 137, which resides in the receptor-binding site (RBS). Two additional residues in the RBS differentiated all (position 226) or most (position 194) of the 1998/9 epidemic strains from the vaccine virus (Fig. 2). The change from I to V at position 226 already become established in the mainstream during the 1996/7 epidemic season, and the change from L to I at

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Additional data	Virus strains	Amino acid positions AAA B BBBB 1111111111112222222 2335934446678899902223 59 3117727249052362487069311
	SDN/5/97	IPNTRKYSISKNDHSTIAKRIRHLD
	MSC/10/99	L.....SR..R.....L.....Y..
a – L	FN/680/99	L...Q.SR..R.....L...V.Y..
a + L	FN/644/99	L...Q.SR..R.....LT..V.Y..
a –	FN/645/99	L...Q.SR..R.....LT..V.Y..
a – L	FN/679/99	L...Q.SR..R.....LT..V.Y..
a + L	FN/686/99	L..AQ.SR..R.....LT..V.Y..
a –	FN/682/99	L...Q.SR..R.....LT..VGY..
a + L	FN/694/99	L...Q.SR..R.....LT.XVXY..
a +	FN/646/99	L...Q.SR..R.....LT..V.Y..
a –	FN/660/99	L...Q.SR..R......T.GV.Y..
a –	FN/681/99	L...Q.SR..R......T.GV.Y..
a +	FN/663/99	L...Q.SR..R......T.GV.YX.
b –	FN/661/99	L.....SG..X...X.L.X.V.Y..
b +	FN/666/99	L.....SX..R...I.L.X.VXY..
b +	FN/662/99	L.....SG.XX.....L.R.V.Y..
b –	FN/665/99	L.....SG......X.L.R.V.Y.N
b + L	FN/678/99	L.....SG.......L.R.V.Y.X
b + L	FN/664/99	L.....SG.......L.R.V.Y.N
b –	FN/659/99	L.....SG.......X.R.VGY.N
b +	FN/656/99	L.....SG.......L.R.VXY.N
b + L	FN/658/99	L.....SG......X.L.R.VGY.N
b +	FN/657/99	L.....SG......I.L.R.VGY.N
b –	FN/684/99	L.....SG.........R.VGY.N
c –	FN/683/99	L.S..RSR...D....L...VKY..
	PNM/2007/99	LS..Q.SRN...EL.IL...V.Y..

Fig. 2. Amino-acid differences in the HA1 domain between 3 reference strains and the 23 field strains from the Sodankylä study population. Arrows refer to RBS. A and B below the arrows refer to antigenic sites A and B. Additional data: a, b and c refer to the phylogenetic subgroups of Figure 1. Vaccination status is indicated by + and −. L refers to reduced (low) reactivity of the particular virus strain in HI tests (Fig. 3). SDN, Sydney; MSC, Moscow; PNM, Panama; FN, Finland.

position 194 has occurred infrequently and shown in association with egg-adaptation [5, 12]. Three subgroup-specific, or nearly specific, amino acid substitutions were recorded during 1998/9 that reside at antigenic sites A or B close to the RBS (Fig. 2). The change from G to R at 142 (site A) is a mainstream change (characteristic of SDN/5/97-like viruses isolated in Finland since their appearance in 1997 [5]) that reverted to G in subgroup b. The change from K to R at 160 (site B) is new, characteristic of subgroup a, and undetected in the previous years in Finland [5, 12]. The change from A to T at 198 (site B), characteristic of subgroup a, was occasionally detected in 1997/8 [5].

Reactivity in HI tests

In preliminary HI typing, all of the 68 influenza A virus strains isolated in Finland during the 1998/9 epidemic season were shown to be H3-subtype strains antigenically related to SDN/5/97 and distinct from the previously circulating variants Beijing/353/89, Beijing/32/92 and Nanchang/933/95 (results not shown). However, a number of field strains showed rather weak reactions to all antisera. The results of a more complete antigenic analysis performed with the 23 isolates from our study population are given in Figure 3. Both major phylogenetic subgroups (a and b) were heterogeneous. Pre- and post-vaccination human serum pools, as well as rat antisera raised against 4 of the 23 MDCK-grown field strains, reacted with most of these strains at titres that did not differ significantly (< fourfold change) from the titres with egg-grown SDN/5/95. However, two types of exceptions were observed. Firstly, the anti-SDN/5/97 pool and post-vaccination serum pool reacted with 7–8 field strains at significantly lower titres than with SDN/5/95. Secondly, antisera raised against the MDCK-grown field strains reacted with 4–7 field strains to significantly higher titres than with the egg-grown SDN/5/95. The reduced reactivity strains

	Virus strains	Antiserum pools 1	2	3	4	5	6	7
	SDN/5/97E	0.9	2.4	**2.4**	1.6	1.8	1.8	1.5
−	FN/680/99M	1.1	1.4	1.3	1.7	1.8	1.7	1.4
+	FN/644/99M	0.7	1.0	1.0	**1.4**	1.6	1.5	1.1
−	FN/645/99M	1.3	2.5	2.2	2.1	2.3	2.3	2.0
−	FN/679/99M	1.0	1.3	1.3	1.6	1.8	1.8	1.4
+	FN/686/99M	1.0	1.4	1.4	1.6	1.9	1.9	1.6
−	FN/682/99M	1.1	2.7	2.5	2.2	2.5	2.4	2.1
+	FN/694/99M	1.0	1.3	1.6	1.8	2.0	1.9	1.6
+	FN/646/99M	1.1	2.5	2.2	2.2	2.3	2.2	1.8
−	FN/660/99M	1.0	2.7	2.8	2.2	**2.5**	2.5	2.2
−	FN/681/99M	1.0	2.7	2.4	2.1	2.3	2.2	1.9
+	FN/663/99M	1.0	2.8	2.8	2.2	2.5	2.4	2.2
−	FN/661/99M	1.0	2.1	2.0	1.9	2.0	**2.2**	1.7
+	FN/666/99M	1.0	2.5	2.2	2.1	2.2	2.2	**1.9**
+	FN/662/99M	1.0	1.9	2.0	1.9	2.2	2.2	1.7
−	FN/665/99M	1.0	2.5	2.5	2.1	2.2	2.2	1.9
+	FN/678/99M	1.0	1.6	1.9	1.6	1.9	1.9	1.5
+	FN/664/99M	0.9	1.5	1.8	1.6	1.7	1.9	1.6
−	FN/659/99M	1.0	2.4	2.4	2.0	2.3	2.3	1.9
+	FN/656/99M	1.0	2.4	2.4	2.1	2.3	2.3	1.9
+	FN/658/99M	0.9	1.4	1.8	1.7	2.0	2.0	1.6
+	FN/657/99M	0.9	2.5	2.5	2.2	2.4	2.5	2.2
−	FN/684/99M	0.9	2.6	2.6	2.2	2.3	2.4	2.1
−	FN/683/99M	1.0	2.5	2.2	2.2	2.2	2.3	2.0

Fig. 3. Haemagglutination inhibition reactions of SDN/5/97 and the 23 field strains from the Sodankylä study population. Strain designation: SDN, Sydney; FN, Finland. E and M refer to isolation and further propagation of the virus in embryonated eggs or in MDCK cell cultures, respectively. Antiserum pools: (1) pre-vaccination sera, (2) post-vaccination sera, (3) anti-SDN/5/97E, (4) anti-FN/644/99M, (5) anti-FN/660/99M, (6) anti-FN/661/99M, (7) anti-FN/666/99M. Geometric mean titres (log) performed in triplicate are indicated. Titres with homologous viruses are in bold face. Underlined titres are ⩾ fourfold lower, and double-underlined titres ⩾ fourfold higher, than the titre of the antiserum in question with SDN/5/97. Vaccination status is indicated by + and −.

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agglutinated goose erythrocytes to somewhat lower titres than the others (GMTs 35 and 46). Six of the 12 strains (50%) isolated from vaccinated patients showed low reactivity, whereas 2 low-reactivity strains were isolated from the 11 unvaccinated patients (18%) ($P > 0{\cdot}19$; 2-tailed Fisher's exact test). Figure 2 indicates that the low reactivity cannot be explained, at least not alone, by differences in particular amino-acid residues of HA1.

Low-reactivity strains were detected in 1999 not only in the study population, but also in some other places in Finland, suggesting that local clusters might occur (results not shown). Some low-reactivity and high-reactivity strains isolated early in 1999 outside the study population were sent to the National Institute of Medical Research in London, where their reactivity was analysed by HI using postinfection ferret sera. The results (Dr Alan Hay, personal communication) were comparable to ours.

DISCUSSION

Despite the better antigenic and genetic match compared with that observed during the previous epidemic season [5], infections with influenza A were frequently detected in our study among vaccinated conscripts during the 1999 outbreak in Finland. Investigation of vaccine efficacy in our study population is in progress. In the USA, a vaccine efficacy of 86% was recorded among healthy adults in the general population.

Viral strains from the study population were similarly heterogeneous for their HA1 sequences and reactivity in HI tests as the strains isolated elsewhere in Finland. Strains exhibiting low reactivity in HI tests were more common in vaccinated than in unvaccinated patients, but the number of isolates was small and the difference not significant. Consequently, virus diversity presumably did not arise in the study population. Low-reactivity strains might be more frequent there than the average level in the general population, but there is no evidence that the higher proportion was due to selection based on enhanced vaccination-induced immunity.

Three variants (subgroups A, B and C) have been described on the basis of their HA1 gene sequences, which circulated widely in the Northern Hemisphere during the 1998/9 epidemic season [14]. Low-reactivity strains were detected at least in subgroups A and B, and the reduced reactivity did not strictly correlate with particular amino-acid changes in HA1 [14]. This correlation was undetected in our study as well, where subgroups a and c represent subgroups A and C, respectively. Subgroup B viruses, which are Panama/2007/99-like and together with other viruses circulated in neighbouring Sweden (Dr Mia Bryttin, personal communication), were not detected in our 1998/9 study in Finland. On the other hand, our subgroup b might have been less widely distributed throughout the world in 1999 than subgroups A, B and C [14]. Low-reactivity strains without correlation to particular changes in HA1 sequences have, to a small extent, also been described during the preceding (1997/8) and following (1999/2000) winters [15–17].

A titre of $\geqslant 40$ (1·6 in our log scale) in HI antibody is frequently referred to as a protective antibody level [18–22]. Our post-vaccination serum pool reacted at a titre lower than this level with 7 of the 23 virus strains isolated in our study population. Five of the low-reactivity strains were isolated from vaccinated patients. If the titre of $\geqslant 40$ is accepted as the protective antibody level, the low reactivity may be advantageous for the virus by allowing it to avoid the vaccine-induced immunity. In garrisons and similar semi-closed communities, vaccination campaigns result in circumstances under which enhanced immunity-mediated selection pressure may be associated with elevated exposure to the virus. Concern may arise that under these circumstances virus variants may become abundant that are capable of breaking through antibody-mediated immunity, even if they would not be as competent in communities characterized by lower selection and infection pressures. We cannot determine if this type of selection occurred in the study population, for low-reactivity strains were also isolated in unvaccinated populations.

MDCK cell-derived H3N2-subtype viruses have been shown to detect HI antibody at higher titres than their egg-derived counterparts [23]. Several amino-acid substitutions near or in the RBS are associated with egg-adaptation [12, 24–27]. Enhanced reactivity of the MDCK-grown field strains and their amino-acid differences in RBS and antigenic sites A and B compared with the egg-grown SDN/5/97, may explain why antisera against the field strains reacted in our HI tests to higher titres with the homologous viruses than with the egg-grown SDN/5/97. The mechanism of the low reactivity, characteristic of several 1998/9 field strains, is a more interesting question yet to be resolved. The low-reactivity strains were shown to agglutinate goose erythrocytes at a somewhat lower titre than did the other field strains.

We suggest that changes or lability in receptor-binding properties may be involved in the low reactivity observed in HI tests, even if correlation to particular amino-acid changes could not be shown.

Several codons associated with the RBS are under positive selection and changed in the 1990s [28]. Mainstream changes in RBS at positions 226 and 190 are shown to be associated with the impaired ability of H3N2-subtype virus to agglutinate chicken erythrocytes [29, 30], and, as an example of 'O' → 'D' phase variation, the change in RBS from A to S at position 138 was shown to restore this ability [31].

Interestingly, substitution from Y to S at the adjacent position 137 was shared by all 1998/9 field strains in the present study. Furthermore, substitution from L to I at position 194, which also has been shown to affect receptor binding [29], was detected in 4 of the 23 strains of our study population. In addition to the recent amino-acid substitutions in the RBS, oligosaccharides present in the new potential glycosylation sites at positions 122 and 133 may also affect receptor binding [33] and be involved in releasing the virus from the receptor, in cooperation with viral neuraminidase (NA) [34, 35]. In Finland these sites were recorded for the first time in 1996/7 and 1997/8, respectively [5]. Sequence analysis of NA genes of the strains isolated in our study population is in progress. Other explanations for the variability observed in our HI tests may include differences in the shape and size of virus particles and the number of their HA and NA spikes.

In conclusion, more knowledge is required concerning the evolutionary significance of the reduced-reactivity virus strains, their underlying mechanisms, and especially their ability in breaking through antibody-mediated immunity. These viruses may be worth monitoring particularly during vaccination campaigns in closed and semi-closed communities, where enhanced immunity-mediated selection pressure may be associated with elevated exposure to the virus.

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