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Evidence of Emergence of New GGII.4 Norovirus Variants from Gastroenteritis Outbreak Survey in France during the 2007-to-2008 and 2008-to-2009 Winter Seasons[▽]

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Analysis of 316 outbreaks of gastroenteritis in France from September 2007 through March 2009 showed that genogroup II.4 (GGII.4) noroviruses were predominant and mostly belonged to the 2006b variant. However, the new GGII.4 variants, variant 2008 and the newly discovered Cairo variant from the Middle East, were also detected. The epidemiological survey suggests that these new variants might become the next predominant strains.

Human noroviruses (NoVs) are causative agents of gastroenteritis worldwide and are increasingly incriminated in outbreaks of gastroenteritis. The molecular typing of NoV is now routinely performed in laboratories involved with surveys of enteric viruses. The recent establishment of networks (e.g., the FBVE and noronet networks) to monitor enteric viruses showed the large predominance of genogroup II.4 (GGII.4) NoVs (11). Over the past 10 years, NoVs and especially GGII.4 variants have increasingly been studied. It has been shown for the first time that GGII.4 NoV variant US95/96 was predominant worldwide during the winter of 1995–1996 (8). NoV variant US95/96 was detected from outbreaks for the next 7 years, until 2002, when a new type of GGII.4 strain, the 2002 variant, named Farmington Hills, was detected and became predominant (7). The main feature of this strain was the insertion of 1 amino acid in the hypervariable region of the capsid (3). Since 2002, a new GGII.4 variant has become predominant every 2 or 3 years, as shown previously. Indeed, the 2002 variant was replaced in 2004 by the 2004 variant (1), which was then replaced by the 2006a and 2006b variants, which cocirculated in 2006 and 2007 (13). In France, the 2006b variant has been predominant since 2008. Previous studies showed that the heterogeneity of the amino acid sequences among GGII.4 variants was no more than 10%. However, Lindesmith et al. showed that minor changes in the capsid between GGII.4 variants were biologically relevant and corresponded to changing patterns in the binding of the glycosaccharides (6).

Moreover, it is worth mentioning that not all GGII.4 variants are circulating throughout the world. For example, the Sakai variants have mostly been detected in Asia. For the Cairo variants that we recently described (4), it was difficult to predict whether they would circulate locally or worldwide. During the last year, the national French survey network and other laboratories in the world detected emerging GGII.4 isolates, called 2008 variants, which might be the new predominant strain. Here we report on the emergence of new GGII.4 isolates, including the 2008 variants, which have been collected and characterized by the French National Reference Center (NRC) for Enteric Viruses.

During a period that included two consecutive winter seasons, from 1 September 2007 through 31 March 2009, 1,422 stool specimens from 316 outbreaks were sent to the NRC for analysis. Sixty-seven percent of the outbreaks were reported during the second winter season (September 2008 through March 2009), thanks to the countrywide improvement in the outbreak reporting system. The NoV outbreaks were reported predominantly from nursing homes (n = 251), but also from hospitals (n = 21), schools (n = 22), and adult meetings (n = 22). RNA was routinely extracted from stool specimens from individuals involved in each outbreak prior to amplification with capsid-specific primers (5). For each outbreak, the PCR products from up to three samples were purified and sequenced for typing, as described previously (4). The presence of at least one virus was detected in 85% of the outbreaks (n = 268), and 254 of these were NoV related. For 14 NoV-negative outbreaks, rotavirus (n = 9), astrovirus (n = 3), sapovirus (n = 1), and Aichi virus (n = 1) were detected by PCR. Twenty-one outbreaks were positive for non-GGII.4 NoVs. The other GGII NoVs belonged to GGII.2 (n = 2), GGII.6 (n = 6), and GGIIb/GGII.3 (n = 4). For nine outbreaks, GGI.2 (n = 2), GGI.3 (n = 3), GGI.4 (n = 1), GGI.8 (n = 2), and GGI.10 (n = 1) were detected. GGII.4 NoV represented the bulk of the strains (229 outbreaks, 72%) (Fig. 1). GGII.4 NoVs were incriminated in 17 of 24 mixed infections. The predominant 2006b variants were identified in 192 outbreaks, 13 of which were mixed infections. The 2006a accounted for 4.7% (n = 14) of

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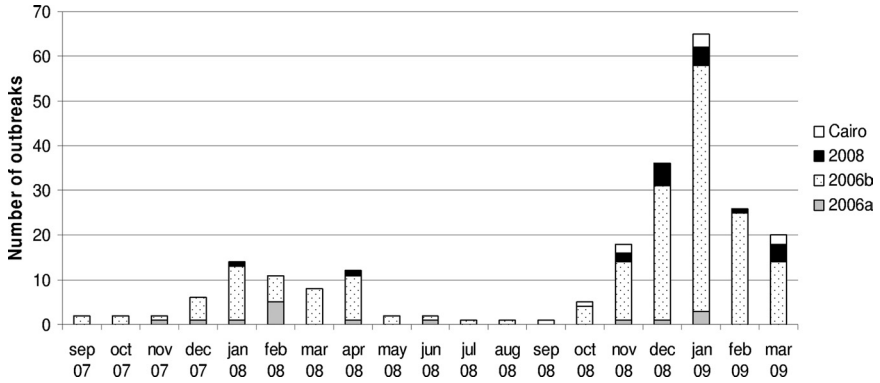

FIG. 1. Monthly distribution of GGII.4 variants during the study period (September 2007 through March 2009). The GGII.4 variants are shaded in gray, and the key is indicated on the right.

the total number of outbreaks (three outbreaks were mixed infections). For 27 outbreaks, the GGII.4 NoVs were not related to other known GGII.4 subtypes. Preliminary typing based on the first 227 bases of open reading frame 2 (ORF2) showed that 9 and 18 of those outbreaks were caused by NoVs that belonged to the Cairo and 2008 variants, respectively (Table 1). Two 2008 variant isolates were documented in January and April 2008, but these variants were mostly detected during the 2008-to-2009 winter season ($n = 16$) (Fig. 1). The Cairo variants clearly emerged in France during the last winter season, with the first outbreak being documented in September 2008. A panel of these variants ($n = 10$) was selected from the typing results obtained by use of the capsid region to undertake sequence analysis of the entire capsid gene. After RNA extraction, the ORF2-coding sequence was amplified by reverse transcription-PCR before it was cloned into the pGEM-Teasy vector, as described previously (4). The entire ORF2 sequence was then determined. Eighty-two sequences (Table 2) corresponding to the complete ORF2 from previously described GGII.4 variants and the sequences from this study (Table 1) were analyzed with the MEGA (version 4) package and the MrBayes (version 3.1) program (9, 12). For the latter, the same settings described previously were used (6), except that the amino acid model was set to "mixed." The amino acid differences mostly occurred in the P2 domain (data not shown). The residues contributing to the attachment of the H type 1 antigen were conserved for the Cairo and the 2008 variants (data not shown) (2). However, the triplet S396-R397-N398, located at the N-terminal part of the P2 domain, was shared only by the 2006b, Cairo, and 2008 variants. Similarly, the triplet D376-A377-N378 was found only for the 2008 variants (data not shown). Interestingly, the threonine inserted at position 395 had been replaced by an alanine residue for all but one of the 2008 variants. Further studies will be required to verify whether these amino acid residues correlate with an increased pathogenicity of the new circulating strains. The trees were generated by the neighbor-joining method by use of the MEGA (version 4) program suite (Fig. 2A) and by Bayesian inference of phylogeny by use of the MrBayes program (Fig. 2B) (9, 12). The topologies of the two trees were similar and showed that the 2008 and the Cairo variants were closely related to the currently predominant 2006b variant (Fig. 2). By combining data from epidemiological surveys with phylogenetic analysis, we clearly showed that during the last season, the emerging variant 2008 and Cairo strains were cocirculating with the predominant 2006b variants (Fig. 1 and 2). It is likely that the cocirculation of variants probably occurred very often in the past, and the increasing efficacies of surveillance

TABLE 1. The 2008 variants and Cairo variants from this study

Isolate[a]	Date of onset (mo/yr)	Setting	Variant[b]	GenBank accession no.[c]
E2271	01/08	Retirement home	2008	
E2763	04/08	Nursing home	2008	
E3020	09/08	Nursing home	Cairo	GQ246791
E3060	10/08	Retirement home	Cairo	
E3138	11/08	Retirement home	Cairo	
E3165	11/08	Nursing home	2008	GQ246792
E3184	11/08	Nursing home	2008	
E3203	11/08	High school	Cairo	
E3367	12/08	Nursing home	2008	
E3379	12/08	Community	2008	GQ246793
E3436	12/08	Retirement home	2008	GQ246794
E3487	12/08	Nursing home	2008	GQ246795
E3550	12/08	Hospital (intensive care)	2008	GQ246796
E3594	01/09	Retirement home	Cairo	
E3642	01/09	Nursing home	2008	GQ246797
E3743	01/09	Nursing home	2008	GQ246798
E3809	01/09	Nursing home	Cairo	
E3880	01/09	Nursing home	Cairo	GQ246800
E3956	01/09	Retirement home	2008	
E3959	01/09	Sporting event	2008	
E4032	02/09	Hospital (geriatric)	2008	GQ246801
E4114	03/09	Hospital (geriatric)	Cairo	
E4126	03/09	Retirement home	2008	
E4134	03/09	Retirement home	2008	
E4141	03/09	Retirement home	2008	
E4158	03/09	Nursing home	Cairo	
E4173	03/09	Retirement home	2008	

[a] Each isolate corresponds to one outbreak.

[b] Typing of GGII.4 NoV variants was based on phylogenetic analysis of partial nucleotide sequences of the capsid region, as indicated in the Materials and Methods section.

[c] Strains for which the complete ORF2 sequence was determined are referenced in GenBank. The corresponding plasmid constructs are available upon request.

TABLE 2. GGII.4 isolates from GenBank used for the analysis

Isolate	Variant	GenBank accession no.	Isolate	Variant	GenBank accession no.
MD145	Bristol	AY032605	E1057	Hunter	EU876890
Camberwell	Bristol	AF145896	0317/NL	Hunter	AY883096
MD134	Bristol	AY030098	Dongen46	Hunter	EF126961
Lordsdale	Bristol	X86557	Nijmegen	Hunter	AB303941
Bristol	Bristol	X76716	Hunter	Hunter	DQ078794
UK317	Bristol	AF414417	DenHaag54	Hunter	EF126962
Symgreen95	US95/96	AJ277619	Ehime	Sakai	AB220923
Yuri	US95/96	AB083781	Guangzhou	Sakai	EF535854
Tiel001	US95/96	AB303922	CR2932	Sakai	DQ419908
Parkroyal	US95/96	AJ277613	Chiba	Sakai	AB220921
Altenkirchen140	US95/96	AF425765	NVgz01	Sakai	DQ369797
DOUG4770	US95/96	AF406793	Sakai	Sakai	AB220922
Oder170	US95/96	AF427114	E1501-Dijon	2006a	EU876894
Miami-Beach	US95/96	AF414424	Cairo 6	2006a	EU876886
Ludwigslust221	US95/96	AF427116	Cairo 3	2006a	EU876883
Burwash Landing	US95/96	AF414425	Cairo 7	2006a	EU876887
Bochum339	US95/96	AY532127	Cairo 9	2006a	EU876889
VA98387	US95/96	AY038600	Cairo 5	2006a	EU876886
Dijon171	US95/96	AF472623	Yerseke	2006a	EF126963
Narita104	US95/96	AB078336	RotterdamP7D0	2006a	AB385639
Grimsby	US95/96	AJ004864	Aomori1	2006a	AB447432
Mora97	US95/96	AY081134	Aomori2	2006a	AB447433
Dillingen 259	US95/96	AF425766	Isumi	2006a	AB294790
Dresden153	US95/96	AY532115	Terneuzen	2006a	EF126964
Beeskow124	US95/96	AF427120	Duan-China	2006b	EU366113
Erlangen195	US95/96	AY532114	Kobe034	2006b	AB291542
Lanzhou	Henry	DQ364459	Cairo 1	2006b	EU876892
Houston-TCH186	Henry	EU310927	E1267-Dijon	2006b	EU876895
Anchorage	Farmington	AY502019	E2703-Dijon	2006b	EU876891
MD-2004	Farmington	DQ658413	E3808-Dijon	2006b	GQ246799
E872-Dijon	Farmington	FJ538900	Nijmegen115	2006b	EF126966
Farmington Hill	Farmington	AY502023	DenHagg89	2006b	EF126965
Apeldoorn317	Farmington	AB445395	SSCS	Cairo	FJ411171
Carlow	Farmington	DQ415279	OC07138	Cairo	AB434770
Langen	Farmington	AY485642	RIS	Cairo	FJ411172
Germanton	Farmington	AY502017	Cairo 8	Cairo	EU876888
CruiseG1	Farmington	AY502020	Cairo 2	Cairo	EU876882
Witney	Farmington	AY588030	Cairo 4	Cairo	EU876884
Oxford B5S22	Farmington	AY581254	Apeldoorn317	2008	AB445395
Oxford B2S16	Farmington	AY587989	Stockholm	2008	AB492092
Chipping Norton	Farmington	AY588028	Erfurt	Outlier	AF427117

systems worldwide are contributing to the detection of emerging GGII.4 variants. The results of our phylogenetic analysis suggest that the 2008 and Cairo variants might have derived from the 2006b variants and support the idea that genetic drift is a major force in the evolution of GGII.4, as described previously (6).

Since 2002, the predominant circulating GGII.4 NoVs have contained an extra amino acid, inserted into the hypervariable region of the capsid, with ongoing genetic drift being observed for this genotype. For the 2007-to-2008 and 2008-to-2009 winter seasons, the 2006a variant and the predominant 2006b variant were circulating in France. During an epidemiological survey conducted in the conurbation of Cairo, Egypt, in 2006 and 2007, we observed that the 2006a variant strains were predominant. We also detected new GGII.4 variants that we called Cairo variants. At this stage, it was unclear whether these new GGII.4 isolates were restricted to the Middle East or could disseminate throughout the world. During the same period, no Cairo variant-like GGII.4 NoV were detected in a large epidemiological survey that we conducted in Tunisia, where only 2004 variants were found (10). The survey of the GGII.4 strains detected by the NRC showed that the 2008 and Cairo variants emerged in France during the 2008-to-2009 winter season. These data show that NoV GGII.4 strains are circulating within Mediterranean countries. For the Cairo variants, we may also hypothesize that new GGII.4 variants might spread from the Middle East to Europe and the Maghreb. Additional studies will be required to further evaluate the circulation of these variants in North Africa. Our data also emphasize the need for a careful survey of enteric viruses that are not routinely screened for by many clinical laboratories.

Plotting of the epidemiological data on the radial tree (Fig. 2) showed that several GGII.4 variants circulated at the same time in the world (e.g., the 2006a, 2006b, 2008, and Cairo variants) and that one type of variant was more often detected locally (e.g., Sakai variants in Asia) or worldwide (e.g., the Farmington Hills variant from 2002 to 2004). Even though the mechanism by which the GGII.4 NoV strains

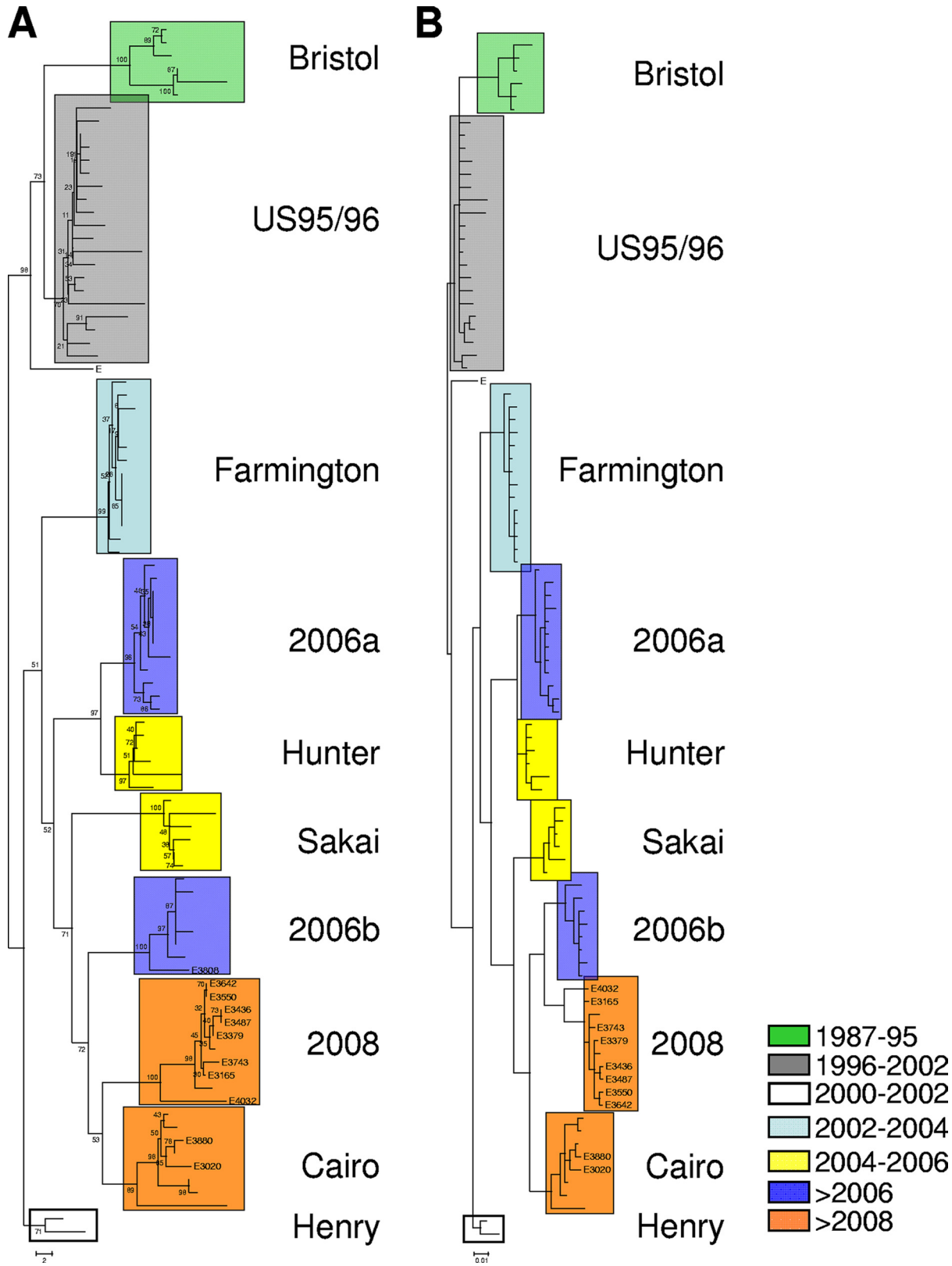

FIG. 2. Phylogenetic analysis of the amino acid sequences corresponding to the entire ORF2 of representative GGII.4 NoVs from GenBank (n = 82) and this study (n = 10). The isolates sequenced for this study are indicated on the tree. The groups of variants are color coded on the basis of the period of circulation of these strains (e.g., the Cairo and 2008 variants) and/or their predominance (e.g., the Bristol, US95/96, 2002, 2004, 2006b, and Sakai variants). The color code is indicated on the bottom right. E, Erfurt outlier isolate (GenBank accession number AF427117). (A) Neighbor-joining tree constructed with noncorrected distances; (B) Bayesian inference tree constructed with previously described settings (6).

evolved remains unknown, the emergence of these new GGII.4 isolates in France might be a precursor to a larger disease burden during coming winter seasons due to these variants.

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