Epidemiological dynamics of norovirus GII.4 variant New Orleans 2009

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Norovirus (NoV) is one of the major causes of diarrhoeal disease with epidemic, outbreak and sporadic patterns in humans of all ages worldwide. NoVs of genotype GII.4 cause nearly 80–90 % of all NoV infections in humans. Periodically, some GII.4 strains become predominant, generating major pandemic variants. Retrospective analysis of the GII.4 NoV strains detected in Italy between 2007 and 2013 indicated that the pandemic variant New Orleans 2009 emerged in Italy in the late 2009, became predominant in 2010–2011 and continued to circulate in a sporadic fashion until April 2013. Upon phylogenetic analysis based on the small diagnostic regions A and C, the late New Orleans 2009 NoVs circulating during 2011–2013 appeared to be genetically different from the early New Orleans 2009 strains that circulated in 2010. For a selection of strains, a 3.2 kb genome portion at the 3’ end was sequenced. In the partial ORF1 and in the full-length ORF2 and ORF3, the 2011–2013 New Orleans NoVs comprised at least three distinct genetic subclusters. By comparison with sequences retrieved from the databases, these subclusters were also found to circulate globally, suggesting that the local circulation reflected repeated introductions of different strains, rather than local selection of novel viruses. Phylogenetic subclustering did not correlate with changes in residues located in predicted putative capsid epitopes, although several changes affected the P2 domain in epitopes A, C, D and E.

INTRODUCTION

Norovirus (NoV) is the most common cause of outbreaks of non-bacterial gastroenteritis and one of the major causes for sporadic cases of acute gastroenteritis in both children and adults (Clark and McKendrick, 2004; Hall et al., 2013; Patel et al., 2008; Tam et al., 2012).

NoV genome possesses three ORFs. ORF1 encodes non-structural proteins including the RNA-dependent RNA polymerase (RdRp), whilst ORF2 and ORF3 encode the major capsid protein VP1 and minor structural protein VP2, respectively (Green, 2013). NoVs can be classified into at least six genogroups, GI–GVI, but only GI, GII and GIV have been found to infect humans (Green, 2013). Genogroups are further classified into genotypes, on the basis of the nucleotide sequences of the RdRp at the 3’ end of ORF1 (polymerase or pol types) and of ORF2 region (capsid or cap types) (Kroneman et al., 2013; Vinjé et al., 2000). Although more than 30 genotypes within genogroup GI, GII and GIV may infect humans (Kroneman et al., 2013), a single genotype, GII.4, has been associated with the vast
majority of NoV-related outbreaks and sporadic cases of gastroenteritis worldwide (Bok et al., 2009). GII.4 strains continuously undergo process of genetic/antigenic diversification and periodically generate novel strains via accumulation of punctate mutations or recombination, with novel GII.4 variants emerging every 2–3 years (Siebenga et al., 2007). Whilst some GII.4 variants such as Cairo 2007, Asia 2003 and Japan 2008 only circulated in limited geographical regions (Kamel et al., 2009; Motomura et al., 2010; Okada et al., 2006), the variants US95/96 1995, Farmington Hills 2002, Hunter 2004, Yerseke 2006a, Den Haag 2006b, New Orleans 2009 (NO2009) and Sydney 2012 were able to spread globally, causing pandemic NoV outbreaks (Eden et al., 2014; Fonager et al., 2013; Siebenga et al., 2009; van Beek et al., 2013; Yen et al., 2011).

The discovery that different NoVs may have different histo-blood group antigen (HBGA) binding patterns and that some individuals are genetically resistant to infection by certain NoV genotypes or variants (Allen et al., 2008; Lindesmith et al., 2008; Ruvoven-Clouet et al., 2013; Shanker et al., 2011) has suggested that difference in human genetic makeup may influence heavily NoV fitness and spread. In addition, most mutations among genotypes and variants occur within five evolving blockade epitopes (A–E) located in the capsid P2 subdomain and surrounding the HBGA binding sites (Eden et al., 2014; Lindesmith et al., 2012, 2013; Zakikhanery et al., 2012). This has suggested that the GII.4 NoV variants undergo antigenic diversification, probably under the pressure of herd immunity (Eden et al., 2014; Lindesmith et al., 2008). Also, in addition to variations in ligand-binding properties and antigenicity, different GII.4 strains exhibit different RNA polymerase rates of mutation (Bull et al., 2010).

In the years 2009–2012, a pandemic GII.4 variant, NO2009, accounted for the majority of NoV outbreaks worldwide (Yen et al., 2011). The variant NO2009 has an ORF1 derived from a Yerseke 2006a parent and an ORF2–3 derived from an Apeldoorn 2008 strain (Eden et al., 2013). Epidemiological studies have revealed that the pre-epidemic ancestral form of the variant NO2009 (Orange 2008, GenBank accession no. GQ845367) was circulating in Australia about 2 years prior to its global epidemic spread (Eden et al., 2010). This pre-epidemic variant was associated with limited local outbreaks and did not demonstrate a tendency to global distribution (Eden et al., 2010; Thornley et al., 2013). Starting from 2012, a new GII.4 variant, Sydney 2012, emerged globally, replacing almost completely the variant NO2009. The ORF1 region of the variant Sydney 2012 was derived from an Osaka 2007 virus, whilst the ORF2–3 region appears to derive from an Apeldoorn 2008 strain (Eden et al., 2013). Interestingly, as soon as the variant Sydney 2012 emerged, intra-genotype recombinant strains with a NO2009 pol and a Sydney 2012 cap started being described worldwide (Fonager et al., 2013; Hasing et al., 2013; Mans et al., 2014; Martella et al., 2013a), posing a challenge for NoV characterization and molecular epidemiology studies.

The Italian Study Group for Enteric Viruses (ISGEV; http://isgev.net) monitors the epidemiology of enteric viruses in children through hospital-based surveillance (De Grazia et al., 2012, 2013; Giammanco et al., 2012, 2013, 2014; Martella et al., 2013a, b; Medici et al., 2014). Data on the prevalence of NoV infection among children in Italy are available uninterrupted at ISGEV since 2007. In the 2007–2013 period, NoV infection was detected in 14.77 % (1027/6950) of children with gastroenteritis (both inpatients and outpatients). In this time span, the most common genotype was GII.4 (384/522, 73.56 %) and five major GII.4 variants emerged consecutively, mirroring the NoV epidemiological patterns described globally. The variant NO2009 appeared in Italy in late 2009, and after April 2013 was no longer detected, suggesting its apparent complete extinction and replacement. Sequence analysis of the small diagnostic regions A and C showed the circulation of several genetic subclusters of the variant NO2009, clearly different from the ‘classical’ NO2009 pol/cap sequence type, starting from January 2011 (Medici et al., 2014). By retrospective analysis and data mining, in the present study we reconstructed the molecular trends and temporal dynamics of the GII.4 variant NO2009 in Italy. The sequence of a large portion (3.2 kb) of the genome at the 3’ end was determined for a selection of GII.4 NO2009 strains representative of the main genetic subclusters identified during routine surveillance.

RESULTS

The pandemic variant NO2009 was detected in Italy as early as October 2009 and continued to circulate in a sporadic fashion until April 2013 (Fig. 1). The variant NO2009 completely replaced the former pandemic variant Den Haag 2006b in early 2010 and was subsequently replaced by the variant Sydney 2012 during 2013.

The partial sequence of ORF1 (807 nt) and the full-length sequence of ORF2 (1621 nt) and ORF3 (849 nt) were determined for 10 Italian NO2009 strains, selected as representative of all the putative genetic clusters identified during the surveillance activity. The sequences were analysed with cognate sequences available in the online databases. No putative recombination break-point events were mapped. Multi-target phylogenetic analysis generated considerable overlaps in the tree topologies (Fig. 2).

In these analyses, the pre-epidemic NO2009 strain Orange 2008 (GenBank accession no. GQ845367) segregated within a separate branch with respect to the pandemic NO2009 reference strain (GenBank accession no. GU445325), exhibiting a nucleotide difference of 1.7 % in ORF1, 2.1 % in ORF2 and 2.1 % in ORF3. All the other NO2009 sequences were grouped into a unique cluster, further divided into four discrete subclusters. One of these subclusters (referred from here on as ‘classical’ NO2009) included the early Italian NO2009 strain, circulating in 2010 (PR6876/2010/ITA) along with
the NO2009 reference strain (GenBank accession no. GU445325). These viruses shared 98.7% nucleotide identity in ORF1, 99% in ORF2 and 99.1% in ORF3. Three additional subclusters, named here as NO2009-a to -c, included NoV strains circulating in Italy during 2011–2013. Five strains, representative of the NoVs circulating in 2011, segregated in subcluster NO2009-a, one strain identified in 2012 was grouped in subcluster NO2009-b, and two strains

Fig. 1. Monthly distribution of GII.4 variants between January 2007 and December 2013.
Fig. 2. Phylogenetic analysis based on partial ORF1 (807 nt) (a), full-length ORF2 (1621 nt) (b) and full-length ORF3 (849 nt) sequences (c) of the Italian GII.4 New Orleans 2009 NoVs (▲). The reference sequences were retrieved from the NoroNet database. The trees were generated using the neighbour-joining method. Bootstrap values were estimated with 1000 replicate datasets and are indicated at each node. Bars, nucleotide substitutions per site.
identified in 2011 and one strain identified in 2013 were grouped in subcluster NO2009-c.

When analysing the pol sequences of inter-pandemic New Orleans 2009/Sydney 2012 recombinant strains detected in Italy during 2012 and 2013 (Fig. 2a), strains PR4200/2012/ITA (GenBank accession no. KF378732) and PR343/2013/ITA (GenBank accession no. KF386146) were grouped in subcluster NO2009-b, whilst strains PA13/2013/ITA (GenBank accession no. KF378731) and PA83/2012/ITA (GenBank accession no. KF378733) were grouped in subcluster NO2009-c.

The Italian NO2009 subclusters a, b and c displayed 1.6–2.1% nucleotide variation in ORF1, 1.5–2.5% in ORF2 and 1.5–2.4% in ORF3 with respect to the ‘classical’ NO2009 strains. Among the three subclusters a, b and c, the nucleotide variation was 1–2.7% in ORF1, 1.5–3.7% in ORF2 and 0.5–2.8% in ORF3. By comparison with GII.4 NO2009 sequences retrieved from the databases, the three subclusters were also found to circulate in European and several countries outside Europe in the same time frame (Fig. 2).

Sequence comparison in the capsid P2 domain (nt 829–1203 of ORF2) showed a 4% nucleotide divergence between the pre-pandemic and the pandemic NO2009 strains, whereas the classical NO2009 strain detected in 2010 (PR6876/2010) and the prototype strain NO2009 shared 99% nucleotide identity. Finally, the three subclusters NO2009-a, -b and -c differed by 1.9–2.7% at the nucleotide level from the prototype strain NO2009.

Upon amino acid sequence comparison, subcluster NO2009-a differed from the prototype strain NO2009 by 0.0–1.1% in ORF1, 0.2–1.3% in ORF2 and 2.1–2.5% in ORF3, whilst subcluster NO2009-b differed by 0.0–2.7%, 0.9–1.5% and 1.1–3.2% and subcluster NO2009-c by 0.0–0.1%, 0.7–1.7% and 1.4–2.1%.

Multiple alignment of the deduced amino acid sequences of the P2 capsid domain of the Italian NO2009 strains was generated and used to find differences among these strains and cognate sequences of reference, parental and ancestral NO2009 strains in putative capsid epitopes A–E. Whilst most amino acid residues appeared to be conserved in the Italian GII.4 NO2009-a, -b and -c strains with respect to the classical NO2009 strains, polymorphisms were observed in the blockade epitopes, at residue 294 (Pro/Ser) in epitope A, residue 340 (Thr/Ala) in epitope C, residue 393 (Ser/Gly) in epitope D, and residues 407 (Ser/Asn) and 413 (Ile/Thr) in epitope E (Table 1). Overall, from one to four amino acid polymorphisms were identified in the blockade epitopes of the P2 domain of the subclusters NO2009-a, NO2009-b, and NO2009-c. Also, in the secondary HBGA/capsid stabilizing interaction site, a change was observed at residue 396 (Pro/His). Some of the amino acid changes detected in NO2009 subclusters anticipated amino acid patterns that are typical of the Sydney 2012 variant (pre-pandemic and reference). These changes were also found in Italian Sydney 2012 subvariants (Giammanco et al., 2014) (Table 1).

Sites within the P2 domain under significant positive selection for the variant NO2009 (Eden et al., 2014) were also analysed (data not shown). Following the subgroup classification proposed by Eden et al. (2014), only two NO2009-a ORF2 sequences (PA278/2011 and PA288/2011) could be classified into an established subgroup (subgroup IV), whilst the other eight Italian strains showed at least five new patterns of amino acid substitutions tentatively indicated as subgroup VI (PR6876/2010), VII (PR11471/2011, subcluster NO2009-a), VIII (PA330/2011 and PA59/2012, subcluster NO2009-a), IX (PA99/2012, subcluster NO2009-b) and X (PR7841/2011, PR9474/2011 and PR328/2013, subcluster NO2009-c).

**DISCUSSION**

By retrospective analysis and data mining of the epidemiological data gathered during Italian surveillance for NoVs, we monitored the activity of the NO2009 variant in Italy, during four epidemic seasons (2009/2010, 2010/2011, 2011/2012 and 2012/2013), confirming that in Italy, as in other European and non-European countries, the NoV variant NO2009 acquired major epidemiological relevance in the years 2009–2012.

Upon molecular analysis of the NoV 3.2 kb genome portion at the 3’ end, discrete sequence variations were observed within the NO2009 strains, allowing distinction of genetic subclusters. The late NO2009 NoV strains, detected in Italy during 2011–2013, were genetically and phylogenetically different from the early strains circulating in 2010 and from the prototype NO2009 strain. Also, the late NO2009 Italian strains of the various subclusters NO2009-a to -c were closely related to NoV strains detected worldwide in the same time frame, suggesting that the circulation of these NO2009 subvariants was not restricted to a single geographical location. These findings are consistent with repeated introductions in the Italian population of different NO2009 strains, rather than with local selection of novel subvariants. The generation of subvariants of GII.4 viruses may be expected during pandemic circulation. Similar evolutionary mechanisms might have been involved in the emergence of the recent pandemic variant GII.4 Sydney 2012. This variant was first identified in 2008 but apparently circulated at low levels until 2012, when it became predominant (Eden et al., 2014). Indeed, we detected the emergence of the variant Sydney 2012 in Italy as early as late 2011 and demonstrated, as observed for the NO2009 subvariants, that these early Sydney 2012 strains were different from the NoV strains endemic in 2012–2013 and from the reference and pre-pandemic Sydney 2012 strains in the capsid epitopes B, C and D (Giammanco et al., 2013; Giammanco et al., 2014). The increased prevalence of the variant Sydney in 2012 has been associated with changes in both ORF1 and ORF2
Table 1. Informative capsid sequence positions of the GII.4 New Orleans 2009 strains belonging to the different subclusters

The putative blockade epitopes A–E and the positions (*) used for subgrouping (Eden et al., 2014) are indicated. Changes in blockade epitopes with respect to the reference strain (in bold) and the early sequence variant GII.4 New Orleans 2009 are underlined.

<table>
<thead>
<tr>
<th>GII.4 Variant</th>
<th>Strain</th>
<th>Subcluster</th>
<th>Blockade epitope/position</th>
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<td></td>
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<td>A</td>
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<td></td>
<td></td>
<td></td>
<td>120*</td>
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<tr>
<td>Apeldoorn</td>
<td>JB-15/2008</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Orange 2008</td>
<td>NSW001P</td>
<td>Pre-epidemic</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>PR876/2010</td>
<td></td>
<td>I</td>
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<tr>
<td></td>
<td>PA278/2011</td>
<td>Late-a</td>
<td>I</td>
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<td></td>
<td>PA288/2011</td>
<td>Late-a</td>
<td>I</td>
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<tr>
<td></td>
<td>PR11471/2011</td>
<td>Late-a</td>
<td>I</td>
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<td></td>
<td>PA330/2011</td>
<td>Late-a</td>
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<td>PA89/2011</td>
<td>Late-a</td>
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<td></td>
<td>PA99/2012</td>
<td>Late-b</td>
<td>I</td>
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<td></td>
<td>PR7841/2011</td>
<td>Late-c</td>
<td>I</td>
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<td></td>
<td>PR9474/2011</td>
<td>Late-c</td>
<td>I</td>
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<tr>
<td>Auckland 2010</td>
<td>PR328/2013</td>
<td>Late-c</td>
<td>I</td>
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<td></td>
<td>NSW 0514/2012</td>
<td></td>
<td>I</td>
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<td></td>
<td>PA363/2011</td>
<td>Early/classical</td>
<td>I</td>
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<tr>
<td></td>
<td>PA48/2012</td>
<td>Early/classical</td>
<td>I</td>
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<tr>
<td></td>
<td>PA703/2012</td>
<td>Late</td>
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with respect to 2008–2010 viruses, as the pandemic GII.4 variant Sydney 2012 arose from a pre-existing GII.4 virus through evolutionary intermediate forms (Bruggink et al., 2014). Similarly, evolutionary analysis has revealed that the ORF2 of the variant NO2009 evolved from the pre-epidemic variant Orange 2008 and thereafter diverged into numerous distinct temporal clusters that mostly coincided with epidemic periods (Eden et al., 2014). A substantial overlapping of the phylogenetic topology was evident in the ORF2 (VP1) and ORF3 (VP2) trees generated in this study, suggesting co-variation of ORF2 and ORF3 in the New Orleans 2009 GII.4 strains, as suggested elsewhere for GII.4 NoVs (Chan et al., 2012; Seah et al., 1999).

Co-circulation of several lineages could be a mechanism to select positive mutations in key residues, generating novel, successful NoV strains. The biological and epidemiological success of GII.4 NoV strains is, in part, influenced by the fixation of mutations in the hypervariable P2 domain, which affects the antigenic profile of the virus (Allen et al., 2008; Lindesmith et al., 2013; Zakikhany et al., 2012), with most variations being observed between different GII.4 variants in five evolving blockade epitopes (A–E). Using virus-like particles with punctate amino acid mutations in the putative capsid epitopes, the binding patterns of blockade mAbs and of convalescent-phase serum collected from GII.4 NO2009 outbreaks have revealed that epitope A is immunodominant and that the GII.4 variant NO2009 has evolved by changing this key blockade epitope (Lindesmith et al., 2013). In addition, epitope A, along with epitope D, has been associated with the antigenic variation observed between the GII.4 variant Sydney 2012 and the former variants Minerva 2006b and NO2009 (Debbink et al., 2013). The evolution in the NoV capsid gene is complex and our understanding of the role of immunity remains limited. The possibility that other mechanisms, as yet unidentified, are also involved remains to be assessed.

It is now believed that the P2 adaptive changes contribute to maintaining and prolonging the circulation of the GII.4 pandemic variants (Eden et al., 2014). The NoV capsid P2 domain contains sites involved in the binding of HBGAs (Lindesmith et al., 2008). The primary HBGA/capsid binding occurs at site I (aa 344–346, 374 and 440–444), whilst a secondary stabilizing interaction occurs at site II (aa 387–396) (Shanker et al., 2011). In the present study a change at site II (aa 396, Pro/His) and some structurally adjacent polymorphisms were found; therefore, HBGA binding variation influencing the spread of NO2009 subvariants cannot be excluded.

In the present study, although the phylogenetic clustering of the NO2009 pre-epidemic, classical and late subvariants did not correlate with specific mutations in the predicted putative capsid epitopes, we identified several amino acid changes affecting the P2 domain at aa 294, 340, 393, 407 and 413. Subvariants (subgroups) within each major GII.4 variant have been defined based on variable sites mostly mapped to the external surface of the P2 domain (Eden et al., 2014). During our surveillance activity, either the contemporary or sequential circulation of as many as four different genetic subclusters and six potential capsid subgroups was documented. Only two of the 10 strains characterized in this study could be classified into the NO2009 subvariant classification proposed by Eden et al. (2014) (subgroup IV), suggesting that this scheme is not adequate to summarize the genetic diversity of the GII.4 NO2009 strains circulating globally.

Circulation of the NO2009 variant partially overlapped temporally with circulation of the Sydney 2012 variant, creating the opportunities for the emergence of inter-pandemic recombinant NoV strains (Martella et al., 2013a). Interestingly, these inter-pandemic recombinant GII.4 strains displayed at least two different pol genes, derived from either the NO2009-b or NO2009-c subvariants, suggesting that they originated from distinct recombination events. The acquisition of various pol types might provide the new variants with the opportunity for acquiring enzymes with higher replication capacity, eventually conferring a better fitness on the mutants (Bull et al., 2010) and posing a challenge for NoV molecular characterization.

In conclusion, the findings of this study indicate that the long time span of circulation of the GII.4 variant NO2009 in Italy was associated with variation in key antigenic epitopes. In addition, the observed changes reflected the results of a process of selection acting on a global scale, rather than the selection and emergence of local strains, as several subvariants already circulating globally were introduced sequentially in the Italian population. Tracking mutations in key blockade epitopes may be important to understand better how GII.4 NoVs evolve and could be necessary in future to refine, over time, the human NoV vaccines and predict the emergence of novel NoV variants.

METHODS

Sample origin. A subset of 522 NoV-positive faecal samples, representing approximately half (50.82 %) of the cases of NoV infection detected by the ISGEV since 2007, was genotyped by sequence analyses in the diagnostic region A (included in ORF1) and C (included in ORF2) and interrogation of the Norovirus Typing Tool database (http://www.rivm.nl/mpf/norovirus/typingtool). Among the 98 NO2009 GII.4 strains detected during the 2007–2013 surveillance, 10 strains were selected as representative of putative genetic clusters on the basis of the analysis of the small diagnostic regions A and C (data not shown).

RNA extraction and amplification. Viral RNA was extracted from 140 μl stool suspension using a QIAamp viral RNA kit (Qiagen). A 3’RACE-PCR protocol was used to generate the 3.2 kb amplicon encompassing the 3’ end of ORF1, full-length ORF2 and ORF3, and the 3’ UTR through the poly(A) tail. Briefly, cDNA was synthesized using a SuperScript III First-Strand cDNA Synthesis kit (Invitrogen) with primer VN3T20 (5’-GAGTGACCGCGGCCGCT20-3’). PCR was then performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe SAS) with forward primer JV12Y (Venemaa et al., 2002) and reverse primer VN1T20 (Wang et al., 2005).
Sequence and phylogenetic analyses. The amplicons were purified and cloned using a TOPOXL Cloning kit (Invitrogen). Additional primers were designed to determine the complete 3.2 kb sequence by an overlapping strategy. Sequence editing and multiple codon-based (translation) alignments were performed with Geneious software v.7.1.7 (Biomatters).

Phylogenetic analysis was performed using the maximum-likelihood method with MEGA v.6.0 (Tamura et al., 2013). Permutation analysis of statistical significance for the reconstructed trees involved 1000 replicates and the same strategy and parameters. SimPlot v.3.2 (Lole et al., 1999) was used to reveal/exclude cross-over sites due to recombination. In addition, the ORF1 sequence of inter-pandemic New Orleans 2009/Sydney 2012 recombinant strains detected in Italy during 2012 and 2013 were included in the analysis. The recombinant strains PR4200/2012/ITA (GenBank accession no. KF378732), PR343/2013/ITA (GenBank accession no. KF386146), PA13/2013/ITA (GenBank accession no. KF378731) and PA83/2012/ITA (GenBank accession no. KF378733) possess a GII.P4 pol type derived from the variant NO2009 and a GII.4 cap type derived from the pandemic GII.4 variant Sydney 2012.

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