The Y155H amino acid substitution in the neuraminidase gene (NA) has previously been associated with highly reduced inhibition by neuraminidase inhibitors in the seasonal H1N1 influenza A virus which circulated in humans before the 2009 pandemic. During the 2012/13 epidemic season in Spain, two A(H1N1) pdm09 viruses bearing the specific Y155H substitution in the NA were detected and isolated from two patients diagnosed with severe respiratory syndrome and pneumonia requiring admission to the intensive care unit. Contrary to what was observed in the seasonal A(H1N1) viruses, neither of the Y155H A(H1N1) pdm09 viruses described here showed a phenotype of reduced inhibition by NAIs as determined by the neuraminidase enzyme inhibition assay (MUNANA). High-throughput sequencing of the NA of both Y155H viruses showed that they were composed to >99% of H155 variants. We believe that this report can contribute to a better understanding of the biological significance of amino acid substitutions in the neuraminidase protein with regard to susceptibility of influenza viruses to neuraminidase inhibitors. This is of critical importance for optimal management of influenza disease patients.

Introduction
Antiviral drugs are essential in the treatment of influenza disease, especially for severe cases, and can play a very important role in the response to the early phases of a pandemic, when a suitable vaccine may not be available and may take several months to develop [1-3]. Due to rapid emergence and spread of strains highly resistant to adamantanes, these first generation antiviral drugs targeting the influenza virus M2 protein, have been replaced by neuraminidase inhibitors (NAIs), oseltamivir and zanamivir [4]. Before their introduction in 1999, analysis of over 1,000 clinical specimens confirmed the general lack of naturally occurring resistance to NAIs [5]. Reduced inhibition by NAIs without treatment pressure was rare in influenza viruses in 2002 [6], although reports of resistance to NAIs associated with treatment appeared subsequently [7,8]. In 2007/08, oseltamivir-resistant seasonal influenza A(H1N1) virus variants associated with the H275Y neuraminidase (NA) mutation (N1 numbering) and unrelated to drug use, emerged and spread globally. During 2008/09, these resistant variants became the predominant seasonal A(H1N1) viruses worldwide [9]. Since late 2009, A(H1N1) seasonal viruses have no longer been in circulation, having been replaced by the swine-origin A(H1N1)pdm09 pandemic virus subtype. The influenza A(H1N1)pdm09 viruses were susceptible to the NAIs oseltamivir and zanamivir, and carried an NA gene segment which belonged to the same genetic lineage as the NA in the Eurasian swine influenza viruses [10]. Since the beginning of the A(H1N1)pdm09 pandemic, oseltamivir resistance has remained but with low prevalence. Several reports describe oseltamivir-resistant A(H1N1)pdm09 viruses bearing the H275Y NA substitution, isolated from patients both treated and untreated with NAIs [11-16], as well as sporadic clusters of patients who acquired infection with these resistant viruses by human-to-human transmission [13,17-19]. These reports raise concerns that the prevalence of viruses exhibiting reduced inhibition by NAIs may increase in the future, and thus, monitoring for the emergence of such viruses is an essential part of national and international surveillance and prevention programmes.
Various amino acid substitutions in the viral NA have been associated with reduced inhibition by NAIs in both seasonal and pandemic A(H1N1) viruses, like the aforementioned H275Y or the E119G/V and I223M/V mutations among others (N1 numbering) [9]. The Y to H substitution in the residue number 155 of the viral NA (N1 numbering) was first described by Monto et al. in a seasonal influenza A(H1N1) virus (A/Hokkaido/15/02 strain) isolated during a study describing the global prevalence of resistance to NAIs during the first three years of their use [6]. Despite the fact that the Y155H substitution is uncommon and has not been included in the main surveillance programmes of the World Health Organization (WHO) [20], it has been listed as a mutation that should be routinely monitored, as recently published by the Antiviral Susceptibility Task Group of the European Influenza Surveillance Network (EISN), Community Network of Reference Laboratories for Human Influenza in Europe (CNRL), coordinated by the European Centre for Disease Control and Prevention (ECDC) [21]. Here we present what is, to the best of our knowledge, the first report of pandemic human influenza A(H1N1) viruses bearing the Y155H amino acid substitution in the NA protein and their susceptibility to NAIs.

Methods

Representative influenza virus isolates and positive respiratory clinical specimens gathered through the Spanish Influenza Surveillance System (SISS) during the 2012/13 influenza season in Spain were sent to our laboratory for complete characterisation. Briefly, the complete haemagglutinin (HA) and NA genes were amplified through RT-PCR and subsequently sequenced following previously described methods [22,23]. We then performed a phylogenetic analysis of the HA sequences to allocate each virus in the different genetic clusters [24]. The sequences of the NA (N1 numbering) were also analysed for the presence of mutations related with reduced inhibition by NAIs (V116A, I117V, E119G/A/V, Q136K, Y155H, D199G/N, I223M/V/K/R, S247N, K262R, H275Y and N295S) [23]. For the sake of clarity, N1 numbering will be used throughout. Selected isolates, including among others those bearing any of these mutations, specimens suspected to have reduced susceptibility to NAIs or from severe cases before start of oseltamivir treatment, were passaged on MDCK cells and their phenotype was analysed using the well described MUNANA assay as recommended in the WHO guidelines [25]. Resistant and sensitive virus controls described MUNANA assay as recommended in the WHO guidelines [25]. Resistant and sensitive virus controls described MUNANA assay as recommended in the WHO guidelines [25]. Resistant and sensitive virus controls described MUNANA assay as recommended in the WHO guidelines [25]).

For the sake of clarity, N1 numbering will be used throughout. Selected isolates, including among others those bearing any of these mutations, specimens suspected to have reduced susceptibility to NAIs or from severe cases before start of oseltamivir treatment, were passaged on MDCK cells and their phenotype was analysed using the well described MUNANA assay as recommended in the WHO guidelines [25]. Resistant and sensitive virus controls described MUNANA assay as recommended in the WHO guidelines [25]. Resistant and sensitive virus controls described MUNANA assay as recommended in the WHO guidelines [25]. The NA gene of the A(H1N1)pdm09 viruses containing the Y155H mutation was amplified and subsequently sequenced with the illumina MiSeq high-throughput sequencing platform. A control virus (A/StPetersburg/100/2011-like) previously characterised in our laboratory as bearing a tyrosine at position 155 was also included. A 1,000 bp NA gene fragment containing the position 155 was amplified through RT-PCR [22,23]. DNA libraries were prepared from the amplicons (starting DNA quantity: 1 ng from each) following the Nextera XT (Illumina) standard protocol. The libraries were sequenced in one illumina MiSeq run of 2x150 (v2) format and represented 7% of the total of samples of the pool which was sequenced. The output was analysed with FastQC and NGStoolkit software for quality assurance. Alignment was performed with BWA alignment software (v.0.6.2) and the variant calling was performed with SAMtools mpileup software (v0.1.18) and a perl script specifically developed for this purpose.

In order to search for other Y155H A(H1N1)pdm09 NA sequences, partial or entire A(H1N1)pdm09 NA sets of nucleotides and amino acid sequences were downloaded from the GISAID Epiflu (http://platform.gisaid.org) database (11,548 nucleotide and 11,871 amino acid sequences available on 2 December 2013). We gratefully acknowledge the originating and submitting laboratories who contributed sequences to that database. Sequences were aligned using Muscle software (v3.8.31). Sequences with a substitution in the residue Y155 were analysed and quantified with an R script specifically developed for this purpose.

Results

Haemagglutinin and neuraminidase genetic analysis

From the beginning of the 2012/13 influenza season until week 20 (2013), we genetically characterised a total of 227 influenza A virus isolates, which were classified according to the phylogenetic analysis of the HA gene sequence (data not shown). Among them, 175 were A(H1N1)pdm09 viruses (143 A/StPetersburg/27/2011-like and 32 A/StPetersburg/100/2011-like). NA gene analysis was performed on 47 A(H1N1)pdm09 viruses, 45 of which did not have any mutations associated with reduced inhibition by NAIs. However, two viruses bearing the Y155H substitution were detected. These two viruses had been isolated from two severely ill hospitalised patients who had been diagnosed with severe respiratory syndrome and pneumonia and required admission to the intensive care unit (ICU) (Figure 1 and Table 1). Influenza A(H1N1)pdm09 infection had been diagnosed in both cases at primary healthcare institutions based on nasopharyngeal aspirates obtained before the start of oseltamivir treatment. The resulting isolates (sample ID 13508 and 13752 from Patient 1 and Patient 2 respectively; Table 1) were then sent to our laboratory for further characterisation. Once we detected the Y155H substitution in both isolated viruses (A/Galicia/508/2013 and A/Extremadura/752/2013 from Patient 1 and Patient 2, respectively; Figure 1), RNA directly extracted from the corresponding nasopharyngeal aspirates was required to confirm the substitution in non-cultured viruses. Original RNA extract...
from the direct sample specimen was only available from Patient 2 but not Patient 1. The Y155H substitution was confirmed in the NA sequence of the original specimen from Patient 2, presenting clear evidence that this substitution had not been generated during primary cell culture isolation.

**MUNANA neuraminidase inhibition assay**

In order to obtain a sufficiently high virus titre to perform the NA enzyme inhibition assay [25], virus isolates from Patient 1 and Patient 2 were passaged in MDCK cell cultures. The sequence of the NA gene of the new viral stocks was analysed again before performing the NA enzyme inhibition assays and the presence of Y155H substitutions was confirmed. NA enzyme inhibition assays were subsequently performed. In those, neither of the two Y155H pandemic viruses showed a phenotype of reduced inhibition by NAIs (Table 2). The IC_{50} values that were obtained for the Y155H viruses, were in the same range as the ones obtained for the other 21 sensitive viruses that were tested in our laboratory during the 2012/13 season (mean IC_{50}: 0.6±0.28, range: 0.17–1.60). In order to confirm these results, viral stocks of both cases were sent to the Respiratory Virus Unit at Public Health England (PHE) in London, United Kingdom. Phenotypic data from both laboratories closely correlated, confirming the absence of reduced inhibition by NAIs in both Y155H A(H1N1)pdm09 viruses (Mean IC_{50} of 0.7 for oseltamivir and 0.4 for zanamivir in the case of A/Extremadura/752/2013, and of 0.3 for both NAIs in the case of A/Galicia/508/2013).

**High-throughput sequencing of the neuraminidase gene of the Y155H viruses**

Original virus isolates from Patient 1 and Patient 2, along with the RNA from the original clinical specimen from Patient 2 and a wild-type control virus (A/StPetersburg/100/2011-like) were included in the high-throughput sequencing analysis. Around 300,000 high quality (70% of the bases with more than 20 Phred quality) reads per sample were obtained from the high-throughput sequencing run. Samples were sequenced to between 30,708 times and 64,154 times average depth of coverage. The first nucleotide of the triplet coding for NA residue 155 was read 35,488 times for A/Galicia/508/2013. Of those, 35,440 (99.86%) contained the codon CAT, coding for a histidine at amino acid position 155, and only 39 (0.11%) contained the wild-type codon TAT, coding for a tyrosine (Sample 508; Figure 2). In the case of A/Extremadura/752/2013, 20,136 reads were obtained for the first nucleotide of the codon of interest, of which 20,124 (99.94%) contained the codon CAT coding for a histidine and 10 (0.049%) contained the wild-type codon TAT, coding for a tyrosine (Sample 508; Figure 2). In the case of A/Extremadura/752/2013, 20,136 reads were obtained for the first nucleotide of the codon of interest, of which 20,124 (99.94%) contained the codon CAT coding for a histidine and 10 (0.049%) contained the wild-type codon TAT (Sample 752; Figure 2). Similar results were observed in the RNA directly extracted from the clinical sample specimen from Patient 2 (Sample 948; Figure 2). In contrast, the wild-type A/StPetersburg/100/2011-like control virus (Sample C077; Figure 2) contained the codon TAT coding for a tyrosine in 35,281 of 35,338 total reads (99.84%). In this sample, 53 (0.15%) reads with the Y155H substitution were also detected. These results correlated with those obtained in the Sanger sequencing (Figure 1) and confirmed that the Y155H viruses can be considered as pure H155 populations.
Influenza A(H1N1)pdm09 viruses with Y155H mutation in the GISAID EpiFlu database

Among 11,871 NA amino acid sequences available at the GISAID EpiFlu database by 2 December 2013, we found 56 sequences with the Y to H substitution at position 155 of the NA. These sequences came from sample specimens collected from year 2009 to 2013 and from different countries in Africa, Asia, Australia, Europe and North America. Information on their phenotype of susceptibility to NAIs was only available for a five of these samples, which had all been reported as not showing reduced inhibition by NAIs in the GISAID EpiFlu database. We also found 10 sequences with a Y to F substitution at position 155, and one with a Y to C substitution.

Discussion

According to data obtained from the EISN network during the 2012/13 influenza season in Europe (up to week 20 in 2013), 11 of a total of 614 A(H1N1)pdm09 viruses tested for antiviral susceptibility carried the NA H275Y amino acid substitution [26]. These 11 viruses were detected mainly in oseltamivir-treated hospitalised patients, some of whom were immunocompromised [26]. Excluding the two isolates bearing the Y155H substitution described here, none of the remaining 601 A(H1N1)pdm09 viruses tested for neuraminidase inhibitor susceptibility showed genetic or phenotypic evidence of highly reduced or reduced inhibition [26]. To the best of our knowledge, this is the first report of the presence of the Y155H amino acid substitution in circulating pandemic A(H1N1) viruses. However, we found 56 NA amino acid sequences with the Y155H substitution among the 11,871 A(H1N1)pdm09 NA records available at the GISAID EpiFlu database by the date of our analysis. These sequences had been reported to the database before and during the 2012/13 season from different countries in Africa, Asia, Australia, Europe and North America. This suggests that A(H1N1) pdm09 viruses containing Y155H had already been circulating worldwide for several seasons, albeit at a very low prevalence.

It should be highlighted that the two influenza A(H1N1) pdm09 viruses with the Y155H mutation were detected in samples obtained before the start of treatment with oseltamivir, and they are thus naturally occurring variants not associated with a selective pressure derived from the usage of NAIs. Monto et al. described for the first time the Y155H substitution in a Japanese seasonal influenza A(H1N1) virus strain (A/Hokkaido/15/02) isolated three years after the licensing of oseltamivir and zanamivir [6]. This strain exhibited a 123- and a 555-fold increase in the mean IC_{50} values for zanamivir and oseltamivir, respectively [6]. However, the two Y155H viruses described here behaved like the sensitive

Table 1

Main clinical characteristics of the studied influenza A(H1N1)pdm09 patients, Spain, 2012/13 (n=2)

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sampling Date</th>
<th>Sex</th>
<th>Age (years)</th>
<th>NAIs Administration</th>
<th>ICU</th>
<th>Risk Factors</th>
<th>Complications</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>13508</td>
<td>24/02/2013</td>
<td>Female</td>
<td>46</td>
<td>Oseltamivir</td>
<td>Yes</td>
<td>Chronic disease</td>
<td>None</td>
<td>Fully recovered</td>
</tr>
<tr>
<td>13752</td>
<td>20/03/2013</td>
<td>Male</td>
<td>49</td>
<td>Oseltamivir</td>
<td>Yes</td>
<td>OSAS</td>
<td>Multi-organ failure</td>
<td>Fully recovered</td>
</tr>
</tbody>
</table>

ICU: intensive care unit; ID: identification number; NAIs: neuraminidase inhibitor; OSAS: obstructive sleep apnoea syndrome.

Table 2

Neuraminidase inhibition of influenza A(H1N1)pdm09 virus isolates, Spain, 2012/13 (n=2)

<table>
<thead>
<tr>
<th>Viral strain</th>
<th>Mean IC_{50} (nM)</th>
<th>NA substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oseltamivir</td>
<td>Zanamivir</td>
</tr>
<tr>
<td>A/Extremadura/752/2013 P2 Siat1</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>A/Perth/265/09 P1 Siat1</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>A/Perth/261/09 P4 Siat1</td>
<td>286.4</td>
<td>1.0</td>
</tr>
<tr>
<td>A/Galicia/508/2013 P3 Siat1</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>A/Perth/265/09 P4 Siat1</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>A/Perth/261/09 P1 Siat1</td>
<td>269.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

IC_{50}: half maximal inhibitory concentration; ISIRV: International Society for Influenza and Other Respiratory Viruses; NA: neuraminidase; P: passage number; Siat1: Siat1 cell line; WT: wildtype.

Data were obtained from two independent MUNANA assays performed at the National Influenza Centre of Madrid, against the ISIRV reference virus panel. Each virus was assayed in duplicate. Data are expressed as mean IC_{50} values in nM. WT denotes isolates not containing any NA substitution related to reduced inhibition by NAIs.

Influenza A(H1N1)pdm09 viruses with Y155H mutation in the GISAID EpiFlu database

Among 11,871 NA amino acid sequences available at the GISAID EpiFlu database by 2 December 2013, we found 56 sequences with the Y to H substitution at position 155 of the NA. These sequences came from sample specimens collected from year 2009 to 2013 and from different countries in Africa, Asia, Australia, Europe and North America. Information on their phenotype of susceptibility to NAIs was only available for a five of these samples, which had all been reported as not showing reduced inhibition by NAIs in the GISAID EpiFlu database. We also found 10 sequences with a Y to F substitution at position 155, and one with a Y to C substitution.

Discussion

According to data obtained from the EISN network during the 2012/13 influenza season in Europe (up to week 20 in 2013), 11 of a total of 614 A(H1N1)pdm09 viruses tested for antiviral susceptibility carried the NA H275Y amino acid substitution [26]. These 11 viruses were detected mainly in oseltamivir-treated hospitalised patients, some of whom were immunocompromised [26]. Excluding the two isolates bearing the Y155H substitution described here, none of the remaining 601 A(H1N1)pdm09 viruses tested for neuraminidase inhibitor susceptibility showed genetic or phenotypic evidence of highly reduced or reduced inhibition [26]. To the best of our knowledge, this is the first report of the presence of the Y155H amino acid substitution in circulating pandemic A(H1N1) viruses. However, we found 56 NA amino acid sequences with the Y155H substitution among the 11,871 A(H1N1)pdm09 NA records available at the GISAID EpiFlu database by the date of our analysis. These sequences had been reported to the database before and during the 2012/13 season from different countries in Africa, Asia, Australia, Europe and North America. This suggests that A(H1N1) pdm09 viruses containing Y155H had already been circulating worldwide for several seasons, albeit at a very low prevalence.

It should be highlighted that the two influenza A(H1N1) pdm09 viruses with the Y155H mutation were detected in samples obtained before the start of treatment with oseltamivir, and they are thus naturally occurring variants not associated with a selective pressure derived from the usage of NAIs. Monto et al. described for the first time the Y155H substitution in a Japanese seasonal influenza A(H1N1) virus strain (A/Hokkaido/15/02) isolated three years after the licensing of oseltamivir and zanamivir [6]. This strain exhibited a 123- and a 555-fold increase in the mean IC_{50} values for zanamivir and oseltamivir, respectively [6]. However, the two Y155H viruses described here behaved like the sensitive
control strains and did not show oseltamivir and zanamivir IC$_{50}$ values in the highly reduced or reduced inhibition range (Table 2). These results are in accordance with the fact that the two patients fully recovered from disease under oseltamivir treatment.

The results of the high-throughput sequencing analysis of the NA of the Y155H A(H1N1)pdm09 viruses revealed that they can be considered as pure H155 populations, as only a very low level of wild-type Y155 background was observed (0.2% of the total reads). These results allow us to discard the possibility that the sensitive phenotype observed in the MUNANA assays for the Y155H viruses could have been conferred by a wild type Y155 background with a higher NA activity, as it has been recently reported for the R292K substitution in H7N9 viruses [27,28].

Therefore, it can be concluded that, in contrast to what was observed for the Japanese seasonal influenza A(H1N1) A/Hokkaido/15/02 strain, a change from Y to H in the residue 155 of the NA does not seem to confer any reduced inhibition by NAIs in A(H1N1)pdm09 viruses. This is somewhat unexpected, considering the significant impact that this substitution has on the A/Hokkaido/15/02 seasonal A(H1N1) virus, as shown by

**Figure 2**

Spectrum of single-nucleotide substitutions at the first nucleotide of the triplet coding for neuraminidase residue 155 of the influenza A(H1N1)pdm09 viruses with Y155H mutation, Spain, 2012/13 (n=2)

CAT: codon for histidine; TAT: codon for tyrosine.

508: A/Galicia/508/2013 Y155H virus; 752: A/Extremadura/752/2013 Y155H virus; 948: RNA extracted from the clinical specimen of Patient 2; C077: wild-type influenza A(H1N1)pdm09 virus (A/StPetersburg/100/2011-like) with a tyrosine at position 155 as obtained from previous Sanger sequencing.

Data are expressed in number of times a nucleotide has been sequenced by independent reads.
McKimm-Breschkin et al. in a recent publication [29]. McKimm-Breschkin et al. show that the Y155H mutation dramatically reduces susceptibility to all NAIs, and that it also reduces plaque size and affects the activity, stability, substrate affinity and pH profile of the NA [29]. However, the different behaviour of our A(H1N1)pdm09 Y155H mutants in the inhibition assays could be explained by distinct structural features of their NA. Qing Li et al. show that the A(H1N1)pdm09 NA does not have the 150-cavity characteristic of other group 1 neuraminidases, including seasonal A(H1N1) viruses [30]. Their results, together with other similar work related not only to NA but also to HA structures, show that the structures of both proteins in the 2009 pandemic virus are distinct from the corresponding structures in the seasonal virus [31,32].

In the case of severe influenza disease, a decision to use the current neuraminidase inhibitors must be made urgently. In these cases it is essential to rule out the presence of a substitution in the NA of the infecting virus which could compromise the effectiveness of the treatment. Our data imply that in a patient with severe influenza disease caused by an A(H1N1)pdm09 virus carrying the Y155H substitution in the neuraminidase, oseltamivir and zanamivir can be chosen as the therapeutic tool. In conclusion, we believe that this report can contribute to a better understanding of the biological significance of amino acid substitutions in the influenza virus NA and HA in relation to susceptibility to NAIs.

GISAID EpiFlu database accession numbers

Sequences from the two A(H1N1)pdm09 viruses described in this manuscript have been submitted to the EpiFlu Database publicly accessible in the GISAID Platform (http://platform.gisaid.org). A/Extremadura/752/2013 (HA: EPI462926, NA: EPI466606). A/Galicia/508/2013 (HA: EPI439740, NA: EPI466205)

Acknowledgements

We would like to acknowledge Dr. Angel Zaballos Sanz at the Genomics Unit of the National Centre for Microbiology of the Carlos III Health Institute and the Next Generation Sequencing service at the Cantoblanco Genomics Unit of the National Centre for Microbiology at the Madrid Scientific Park for their support, and all the members of the Spanish Influenza Surveillance System working in the identification and declaration of patients during the 2012/13 influenza season. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID EpiFlu Database which have been included in this research. This work was supported by Instituto de Salud Carlos III (Programa especial de investigación sobre la gripe pandémica) G009/0040. Sara Monzon received a research contract funding by the Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER U758). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflict of interest

None declared.

References


Authors’ contributions

Unai Perez-Sautu, Francisco Pozo and Immaculada Casas designed the study and performed the genotypic characterisations, the phenotypic neuraminidase enzyme inhibition assays (MUNANA) and the sample preparation for the high-throughput sequencing assay, as well as the interpretation of all data. Unai Perez-Sautu wrote the article, which was revised by Francisco Pozo, Immaculada Casas and Angie Lackenby. Isabel Cuesta, Sara Monzon and Unai Perez-Sautu performed the high-throughput sequencing and bioinformatics data analysis. Ana Calderon, Monica Gonzalez and Mar Molinero provided technical assistance with the RT-PCR methods, sequencing and MUNANA assays. Angie Lackenby confirmed the MUNANA results at Public Health England, London, UK. Isabel Lopez-Miragaya, Sonia Rey, Angelina Cañizares, Guadalupe Rodriguez and Carmen Gonzalez-Velasco provided all the clinical samples and gathered and provided all the relevant clinical data.

www.eurosurveillance.org


