Rapid spread of the novel respiratory syncytial virus A ON1 genotype, central Italy, 2011 to 2013

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Respiratory infections positive for human respiratory syncytial virus (RSV) subtype A were characterised in children admitted to hospitals in Rome and Ancona (Italy) over the last three epidemic seasons. Different strains of the novel RSV-A genotype ON1, first identified in Ontario (Canada) in December 2010, were detected for the first time in Italy in the following 2011/12 epidemic season. They bear an insertion of 24 amino acids in the G glycoprotein as well as amino acid changes likely to change antigenicity. By early 2013, ON1 strains had spread so efficiently that they had nearly replaced other RSV-A strains. Notably, the RSV peak in the 2012/13 epidemic season occurred earlier and, compared with the previous two seasons, influenza-like illnesses diagnoses were more frequent in younger children; bronchiolitis cases had a less severe clinical course. Nonetheless, the ON1-associated intensive care unit admission rate was similar, if not greater, than that attributable to other RSV-A strains. Improving RSV surveillance would allow timely understanding of the epidemiological and clinicopathological features of the novel RSV-A genotype.

Introduction

Human respiratory syncytial virus (RSV) is a major cause of lower respiratory-tract infection (especially bronchiolitis) in infants, but also affects immunocompromised and elderly patients [1]. In a small proportion of infected infants, severe RSV disease is characterised by wheezing, hyperinflation, atelectasis, increased mucus secretion, tachypnoea, retraction and consolidation. RSV, a *Pneumovirus* of the *Paramyxoviridae* family, has an enveloped, non-segmented, single-stranded, negative-sense RNA genome of approximately 15,000 nt, coding for 11 genes. It has been further divided into subtypes A and B [2] based on reactions with monoclonal antibodies against the glycoproteins G and F. The attachment glycoprotein G is a transmembrane glycoprotein with an extracellular domain that consists of two hypervariable regions separated by a central conserved region spanning amino acids 151–190, comprising the receptor binding site [3]. Although the amino acid sequence of the hypervariable region can vary widely between subgroups and even among isolates, its amino acid content is reminiscent of mucin proteins [3]. These mucin-like domains present different N- and O-linked glycosylation sites which may affect antigenicity [3]. Indeed, G is the major antigenic protein of RSV; besides exhibiting marked genetic variability in the amino acid sequence, position and number of glycosylation sites, it may also differ in length due to insertions and mutations in premature stop codons [3]. The main genetic change detected to date is a 60 nt insertion in G (generating a 20 aa insertion in the C-terminal domain) in RSV-B strain BA [4].

Based on the genetic diversity of glycoprotein G, RSV-A has been classified into genotypes GA1–GA7, SAA1 and, recently, NA1–NA2 [5-7], and RSV-B into GB1–GB12, SAB1–SAB3 and BA [4-6]. Most molecular epidemiological studies have analysed the G gene's hypervariable C-terminal domain; importantly, sequencing of the entire RSV genome of several circulating strains [8] confirmed that the evolutionary patterns and clades seen in the C-terminal part of the G gene reflect those of the whole genome. Phylogenetic analysis documented that several C-terminal positions are positively selected hypervariable sites that may contribute to immune escape, promoting re-infection and recurrent circulation of a genotype [9,10]. Multiple genotypes can co-circulate during successive epidemic seasons, but a new subtype, or one spreading from different countries, may replace dominant strains [7,8,11]. Genotype BA spread slowly and sequentially worldwide [12], but over several years, a divergent BA lineage replaced all RSV-B genotypes [13,14].

Aside from the epidemiological impact, novel variants may display enhanced clinical severity with increased replication in the lower respiratory tract and/or hyperresponsiveness of the airways [1,15], underlining the importance of monitoring their spread.

In Italy there is no ongoing national surveillance for circulating RSV, and only one study has investigated the genetic diversity of RSV-A in different seasons, up to 2006 [16]. In that and in other European studies [12,17], phylogenetic analysis disclosed co-circulation of genotypes GA2 and GA5 from 1998 to 2006/07; GA7 was less common.

Within the framework of an ongoing study of paediatric respiratory infections, we genotyped RSV-positive cases detected in Rome and Ancona, central Italy, in the 2011/12 epidemic season. Unexpectedly, we detected ON1, the new RSV-A variant of GA2 genotype identified in Ontario (Canada) in 2010 [18]. ON1 bears a 72 nt insertion in the G hypervariable region, corresponding to 24 aa (of which 23 are duplicated), the largest G protein genetic modification ever reported [18]. The ability of the G protein to host long inserts without impairing function is due to the relatively loose structural constraints of the mucin-like domain, which determines rapid evolutionary changes and contributes to the pathogenicity of RSV and other negative-stranded RNA viruses encoding this domain [19].

In this study, phylogenetic analysis of the G gene of RSV-A strains circulating in the 2010/11, 2011/12 and 2012/13 epidemic seasons demonstrated the presence of ON1 genotype in the 2011/12 winter and its rapid spread in the following year. Investigating the spread of ON1 is important to understand the extent to which genetic variability can modify the epidemic behaviour of RSV at population level. Moreover, since antigenic variation may influence clinical outcomes, we also addressed the clinical impact of genotype ON1. Analysis of case distribution and clinical patient data showed differences between infections with ON1 genotype and those with previously circulating RSV-A strains.

Methods

Patients and samples

The study involved RSV-positive respiratory samples collected in the 2010/11, 2011/12 and 2012/13 epidemic seasons from two sets of patients: children presenting to the paediatric emergency department who were admitted for respiratory conditions and then diagnosed at the virology laboratory of the teaching hospital Policlinico Umberto I-Sapienza University of Rome (RM samples), and children admitted to the paediatric department of Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona-Università Politecnica delle Marche, or to other paediatric departments of the Marche region that use the Ancona virology unit (AN samples). The two cities lie about 200 km apart in central Italy.

Informed consent was sought from the children's parents for participation in the study which had been approved by the ethics committee of the two hospitals.

Demographic and clinical data were taken from the medical files. A nine-point (0–8) clinical severity score based on (age-adjusted) respiratory rate, arterial oxygen saturation in room air, retraction and oral feeding ability were determined on admission [20].

RSV detection

Each institution used their own protocol to test upper respiratory tract samples for respiratory viruses as detailed below. Bronchoalveolar lavage (BAL) samples were taken from patients admitted to a paediatric intensive care unit (PICU) for severe respiratory conditions. In Rome, nasal washings and/or BAL were tested for 14 respiratory viruses with PCR-based tests, as described previously [20,21]. In Ancona, nasal swabs and/or BAL were first tested for RSV RNA using an in-house one-step multiplex real-time RT-PCR followed by further analyses with the Seeplex RV 15 ACE Detection Kit (Seegene, Korea) in RSV-negative samples. Information on PCR primers and probes is available from the authors on request.

RSV-A sequencing

About half of the RSV-A-positive samples were randomly selected for genomic characterisation. Amplicons (502 bp) for sequencing were obtained from RSV-A-positive samples with the A-Fseq (G gene position 481–498 of the RSV-A2 reference strain) and the F1 reverse primer targeting the fusion protein gene's 5' end [5]. Experimental details are available from the authors on request.

Phylogenetic analysis

The nucleotide sequences of a fragment of the second hypervariable region of the G gene (396 nt, corresponding to codon positions 167–298) from RSV-A isolates were determined and compared with reference strains in GenBank.

Sequences were edited using Bioedit v7.1.3 and aligned with reference sequences using CLUSTAL W. We analysed evolutionary relationships between the study sequences, and sequences recently circulating in the United States [8], Canada [18], Belgium [22], Japan [7] and Malaysia [23] together with reference strains representative of all RSV-A genotypes. The best-fit evolutionary model and parameters were selected using jModeltest v0.1.1 [24]. The general time reversible +G (GTR+G) model of nucleotide substitution was the most appropriate for the dataset. The evolutionary parameters corresponding to the best-fit model were run in MEGA5 v5.2.1 [25] to obtain the distance matrix among groups, the tree topology under a strict maximum likelihood (ML) approach, and the significance of the tree topology by bootstrapping (1,000 replicates); the p-distance among sequence clusters was calculated by pairwise comparison including transitions and transversions.

Sequences were submitted to GenBank and assigned the following accession numbers: KC858158–KC858194

and KC858195–KC858198 (AN and RM sequences; 2010/11); JX988439–JX988449, JX988453–JX988486 and JX988450–JX988452, JX988487–JX988499 (AN and RM sequences with/without the 24 aa insertion; 2011/12); KC858199–KC858245 and KC858246–KC858257 (AN and RM sequences; 2012/13).

Statistical analysis

The Mann–Whitney U test was used to compare median patient age, Fisher's exact test to analyse independent categorical variables and the unpaired t-test to compare genetic distance group means. A p value of ‹0.05 was considered significant. SPSS (v17.0) was used for data analysis.

Results

RSV-positive patients

From November 2010 to May 2013, 515 RSV-positive patients were detected: 165 in Rome (mean age: 4.8 months; median age: 2.75 months; range: 0.2–29 months) and 350 in Ancona (mean age: 12.8 months; median age: 3 months; range: 0.1–163 months). Their distribution is reported in Table 1: 180 cases in 2010/11 (83 RSV-A, 97 RSV-B), 65 cases in 2011/12 (119 RSV-A, 46 RSV-B) and 170 cases in 2012/13 (158 RSV-A, 12 RSV-B). All patients were hospitalised for respiratory conditions.

Of the 360 RSV-A positive samples, 161 were successfully sequenced and categorised in relation to the presence of a 72 nt insert in the G gene (Table 2).

RSV case distribution

Analysis by week of presentation highlighted a different case distribution in the last season both in Rome and Ancona (Figure 1). In 2010/11 and 2011/12, the earliest RSV-associated hospitalisations occurred in mid-December (slightly earlier in Rome) and peaked in January and February; in 2012/13, the cases started in late November, with a larger number in both cities occurring earlier than in the previous two seasons, and peaking in week 51 (2012) and week 1 (2013) in Rome and Ancona, respectively. In 2012/13, RSV-A accounted for 93% of all cases.

Table 1

Number of respiratory syncytial virus-positive patients diagnosed at two institutions in Ancona and Rome during three epidemic seasons, Italy, November 2010–May 2013 (n=515)

Winter seasons	RSV-A cases n=360		RSV-B cases n=155		All n=515
	Ancona	Rome	Ancona	Rome	
2010/11	53	30	73	24	**180**
2011/12	79	40	34	12	**165**
2012/13	103	55	8	4	**170**

RSV: respiratory syncytial virus.

Table 2

Sequence features of respiratory syncytial virus-A-positive strains in relation to the presence of a 72 nt insert, Italy, November 2010–May 2013 (n=161)

Winter seasons	Non-ON1[a] n=95		ON1[b] n=66		All n=161
	Ancona	Rome	Ancona	Rome	
2010/11	37	4	0	0	**41**
2011/12	34	13	11	3	**61**
2012/13	7	0	40	12	**59**

RSV: respiratory syncytial virus.

[a] RSV-A genotype without the insertion.

[b] RSV-A genotype with the insertion.

ON1 detection

Phylogenetic reconstruction of 85 unique sequences is reported in Figure 2. All AN and RM strains were derived from genotype GA2 and belonged to the recently described NA1 group [7,23]. They clustered into several distinct clades which, despite non-significant bootstrap values, reflect RSV-A variability and evolution during the three epidemic seasons. The major finding was that 51 AN and 15 RM strains grouped with the novel genotype ON1 (ON67-1210A) [18], which is characterised by a 72 nt insertion in G, resulting in 24 extra amino acids of which 23 are duplications of aa 261–283. ON1 was not detected in Ancona or Rome in the 2010/11 season; it accounted for 14 of 61 (22.9%) strains sequenced in 2011/12 and for 52 of 59 (88.1%) strains analysed in 2012/13. In that last season. it was the prevalent genotype in Ancona (40 ON1/47 RSV-A) and apparently the sole genotype in Rome (12/12).

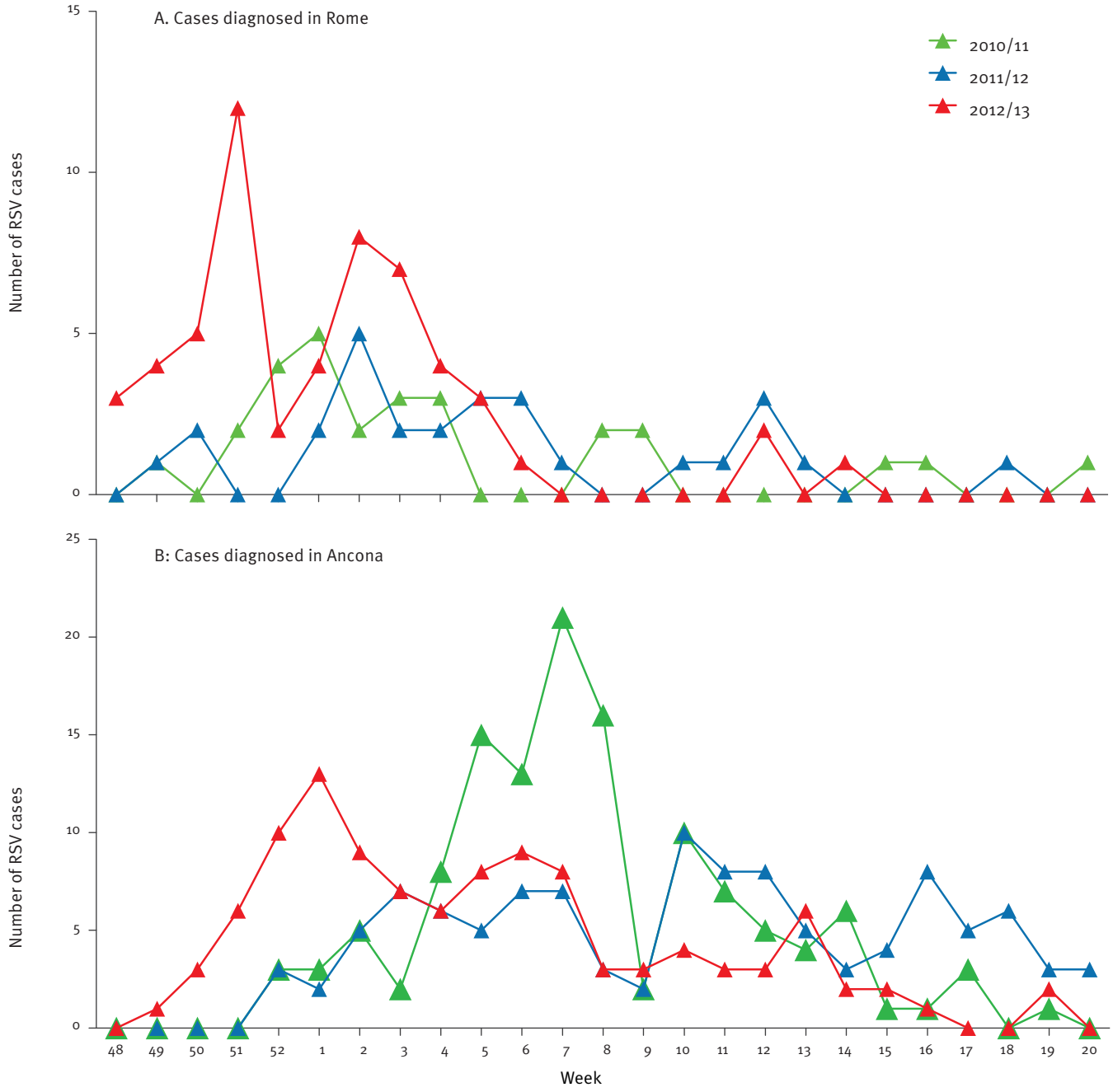
Sequence analysis

The phylogenetic tree based on G gene sequences (Figure 2) shows, next to the main branches, the amino acid substitutions identifying a subtree. At the nucleotide level, the mean p-distance among the 95 strains without the insertion (GA2; NA1 group) was greater than the one among the 66 strains bearing it (ON1), i.e. 0.022±0.016 vs 0.012±0.009 (mean±standard deviation, p‹0.0001, unpaired t-test), suggesting a more recent common origin of the ON1 strains. As expected in this highly variable region, pairwise distances were higher at the amino acid than at the nucleotide level, and were 0.043±0.030 and 0.028±0.022 among strains without and with the insert, respectively (p‹0.0001, unpaired t-test). The alignment of deduced amino acid sequences is presented in Figure 3, with grey areas representing potential N-glycosylation sites.

Overall our strains presented very few amino acid substitutions in the conserved central portion (up to aa 198) of the G protein, whereas, as expected, several variations with respect to the reference GA2 sequence were found in the hypervariable portion.

Figure 1

Respiratory syncytial virus cases, distribution of hospitalised children, Italy, November 2010–May 2013 (n=515)



RSV: respiratory syncytial virus.
Results are weekly data for each epidemic season.

All study sequences clearly differed from the reference GA2 genotype, as demonstrated by the R204K, L215P, S230P conserved substitutions, also found in recently circulating strains of GA2 group, i.e. the NA1, NA2 and ON1 genotypes. Importantly, amino acid positions 215 and 230 are highly variable, positively selected sites [10]. Moreover, all study strains bore the N297K substitution and all but one (17294AN, detected in 2011/12) also exhibited substitution P292S, another positively selected site [10]. Most AN and RM sequences bore the P274L substitution (a positively selected site) that had been detected in NA1 variants in Belgium, Wisconsin, Japan and Malaysia, but not in nine 2010/11 and in one 2011/12 sequences analysed in this study. The substitutions I208L and N273Y/H/D were also found. Changes at position 273 are particularly interesting as they involve the loss of a potential glycosylation site; they occurred in 18 strains from 2010/11), 23 strains from 2011/12 and six strains from 2012/13.

The N273Y substitution was also conserved in the well-defined clade containing 14 (2011/12) and 53 (2012/13) study strains with ON1 genotype (ON67-1210A). This cluster displayed the characteristic 72 nt duplication and three amino acid variations E232G, T253K and P290L, the latter a reversal mutation compared with all other study sequences. Importantly, T253K is related to the loss of another potential N-glycosylation site besides that determined by N273Y; the loss of two potential N-glycosylation sites is a major characteristic of strain ON67-1210A [18]. Moreover our ON1 strains presented several amino acid substitutions in the hypervariable portion of the G protein and in the 24 aa insert compared with the ON1 prototype (Figure 3). When amino acid sequences of the duplicated tract were compared with the homologous 23 aa portions (aa 261–283), variations from the ON1 prototype were generally found either in the insertion or in its homologous tract (Figure 3), suggesting that they arose after the insertion event. Interestingly, however, a reversion in the positively selected site 274 (L274P) was found in a single 2011/12 strain (12221AN) and in 35 of 52 (67%) 2012/13 strains (both AN and RM) in the insert or in the homologous 23 aa portion (L274P and/or LxvP), nearly always together with the YxxiH change in the insert.

Patient data and clinical diagnosis

Demographic and clinical data were available for 99 patients infected with RSV-A-positive strains sequenced in 2011/12 and 2012/13; data stratified by RSV strain are reported in Table 3.

Overall, children whose RSV strain bore the insertion were significantly younger than those infected with the other strains, they had less frequently bronchopneumonia and more frequently influenza-like illness (ILI). ON1 infection caused more, although not statistically significant (p=0.053), PICU admissions than the other RSV-A strains (Table 3).

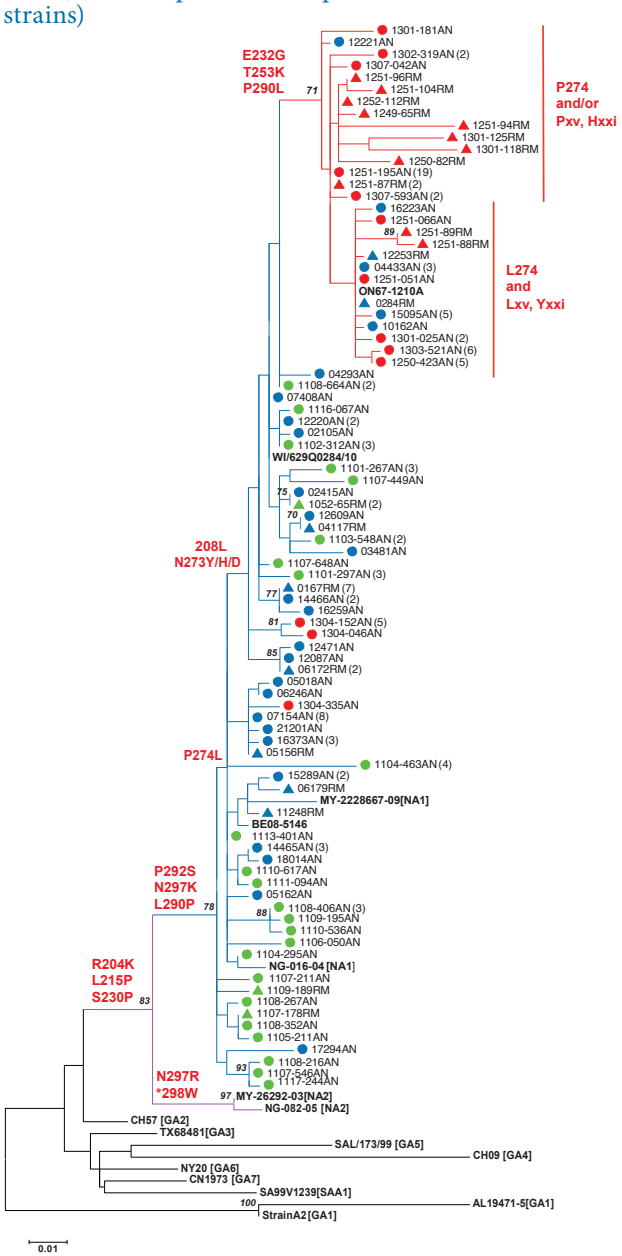
We then evaluated 52 bronchiolitis patients for whom clinical data were exhaustive (Table 3); PICU cases were excluded due to insufficient clinical information and lack of an overall assessment of risk factors for severe RSV disease. Patients positive for an RSV-A strain that bore the insertion were less likely to have an elevated respiratory rate and exhibited a lower, although not statistically significant, clinical severity score (Table 3).

Discussion

It has been estimated that RSV infects 70% of children during their first year of life and that nearly all two year-olds have been infected; in addition, more than one third of children younger than two years get infected at least twice with RSV strains not only heterologous at the subtype level (i.e. RSV-A and RSV-B), but also homologous (i.e. RSV-A and RSV-A or RSV-B and RSV-B) [1,26]. It is not entirely clear whether this is because RSV infection does not confer long-lasting protective immunity in humans, or because recurrent infections

Figure 2

Phylogenetic tree based on the second hypervariable region of the G protein gene, Italy November 2010–May 2013 (n=85 unique Italian sequences, n=16 reference strains)



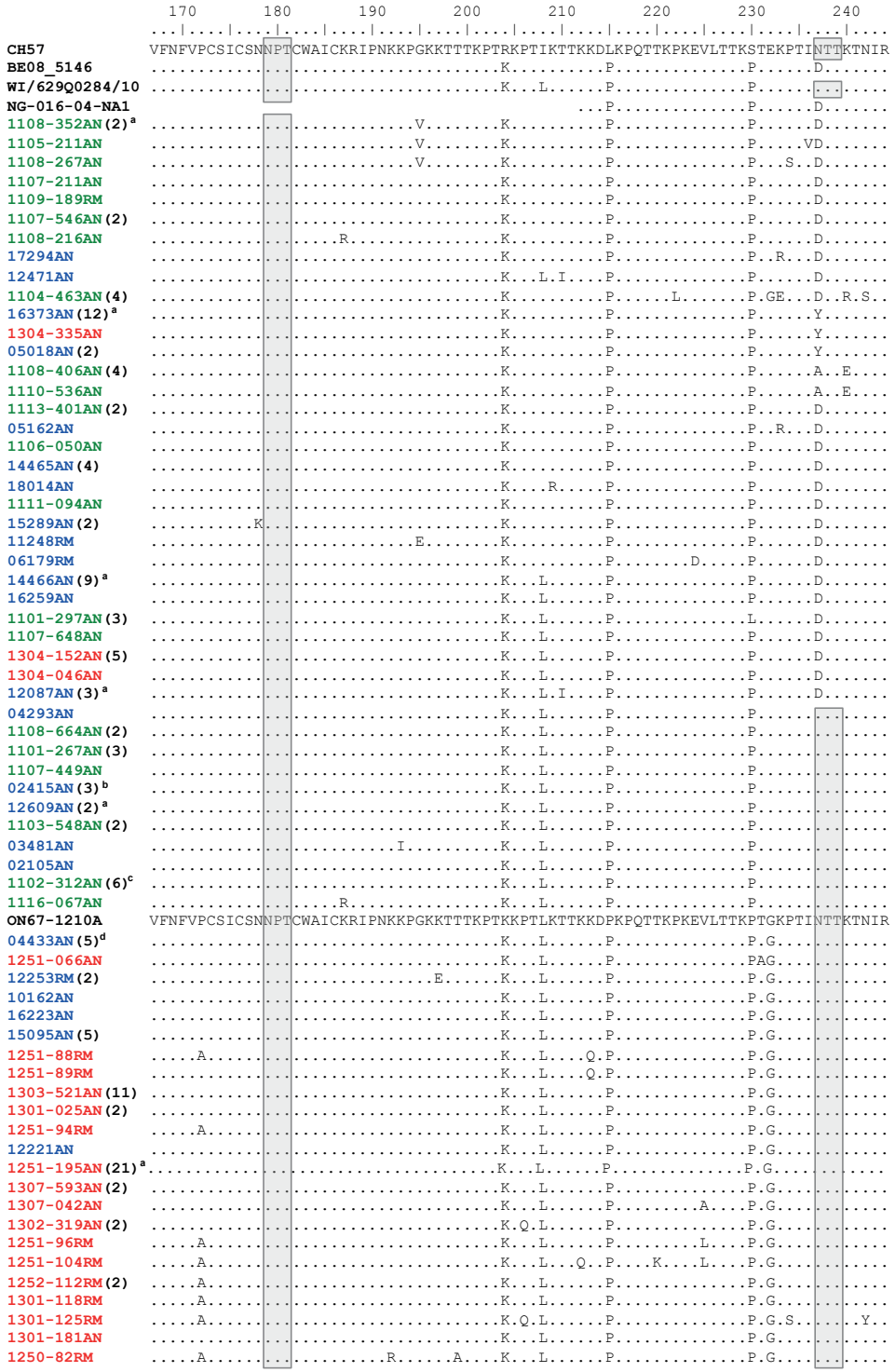
The best-fit evolutionary model and the parameters selected by the jModeltest programme were used as described in the text. The scale bar shows the proportions of nucleotide substitutions per site. Numbers at nodes are bootstrap values for 1,000 iterations; only bootstrap values of >70% are shown. Numbers in round brackets indicate the total number of strains with an identical sequence. Names in square brackets indicate the genotype of the RSV-A reference sequence.

Branches are colour-coded according to the deduced amino acid sequence, identifying subtrees and genotypes: red: sequences with insertion clustering with the novel ON1 genotype; blue: sequences of NA1 genotype clustering with sequences from the United States (Wisconsin) [8], Europe (Belgium) [22], Malaysia (Kuala Lampur) [23] and Japan (Niigata City) [7]; purple: sequences of NA2 genotype from Malaysia (Kuala Lampur) and Japan (Niigata City), added for clarity. Circles: strains isolated in Ancona; triangles: strains isolated in Rome. Symbol colour indicates epidemic season: green: 2010/11; blue: 2011/12; red: 2012/13.

GenBank accession numbers of RSV strains (this study) are reported in the text; reference strain accession numbers are: ON67-1210A (JN257693), WI/629-Q0284/10 (JF920053), MY-2228667-09 (JX256883), BE08-5146 (JX015499), NG-016-04 (AB470478), MY-26292-03 (JX256960), NG-082-05 (AB470479), CH57 (AF065258), TX68481 (AF233920), SAL/173/99 (AY472094), CH09 (AF065254), NY20 (AF233918), CN1973 (AF233904), SA99V1239 (AF348808), AL19471-5 (AF233902), strain A2 (M74568).

Figure 3

Alignment of deduced G protein amino acid sequence of RSV-A strains isolated in Ancona and Rome, Italy, November 2010–May 2013 (n=161)



[a] Strain observed in AN and RM.

[b] Strain observed in AN and RM, in 2010-11 and 2011/12.

[c] Strain observed in 2010/11 and 2011/12.

[d] Strain observed in AN and RM in 2011/12 and 2012/13.

Alignments of 65 unique sequences are shown relative to the sequence of the prototype GA2 CH57 (AF065258) used as the reference strain. Reference sequences belonging to the NA1 subgroup of the GA2 genotype circulating in Europe (BE08-5146, JX015499), the United States (WI/629-Q0284/10, JF920053) and Japan (NG-016-04, AB470478) are included for clarity. Amino acid numbering from 167 to 298 refers to the G glycoprotein of the reference strains without insertion, Roman numerals indicate the position in the amino acid insertion. The ON67-1210A (ON1 genotype, JN257693) sequence with the 24 aa insertion (23 of which are duplicated) is used as the prototype for the Italian strains with the insertion. Dots indicate nucleotides identical to the CH57 strain; the sequence of the ON1 prototype is shown only for clarity. Dashes indicate the gap corresponding to the nucleotide insertions, asterisks indicate stop codons. Black boxes indicate the duplicated region (homologous portion and insertion); sequences within the box are shown independently of identities to the ON1 prototype, mutations are shown in red and bold. Grey shading indicates potential N-glycosylation sites. Colour of sequence names indicates the epidemic season: green: 2010/11; blue: 2011/12; red: 2012/13. Numbers in parentheses indicate the total number of identical strains.

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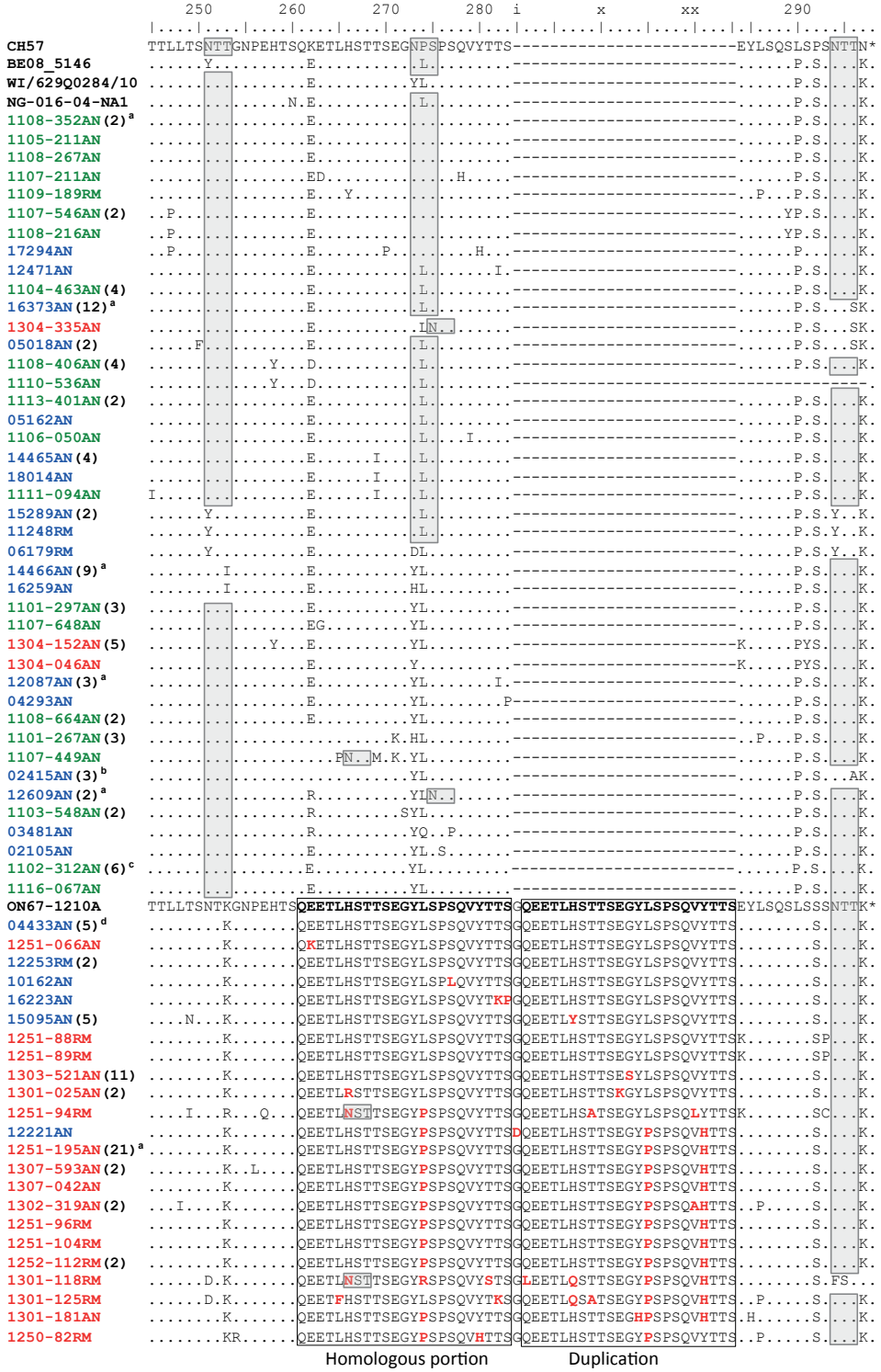

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even with the same subtype are due to significant antigenic changes in the immunodominant proteins [1,26].

Emerging RSV variants that possess a selective advantage in terms of genetic diversity can spread to neighbouring areas, gradually replacing dominant genotypes over several years [7,11,22]. A well- studied example is a 20 aa insertion in the G protein (comparable in length and position to the insertion in ON1) that arose in RSV-B BA strains; it was first detected in 1999 in Argentina [4] and spread worldwide in the course of several epidemic seasons [12-14,22]. In apparent contrast to its epidemiological success, a BA strain was efficiently neutralised by sera from patients previously infected with non-BA RSV-B [27]. However, homologous subgroup reinfections were reported with both RSV-A and RSV-B strains [26,28].

Our findings document the presence of the novel ON1 genotype in Italy in the 2011/12 epidemic season and its rapid spread in 2012/13. All other RSV-A G-gene variants described here derive from the GA2 genotype and are genetically close to the recently characterised NA1 genotype, as are most recent strains circulating worldwide [18,23,28].

The ON1 genotype was first detected in Ontario in the winter of 2010/11 [18], then in 2011/12 season in South Africa [29], Malaysia [23] and Germany [30], with an infection rate of around 10% among hospitalised children. Compared with the near absence of ON1 infections in central Italy in 2010/11, we here report a 23% rate in 2011/12 and, remarkably, a nearly 90% rate in 2012/13, i.e. a nearly complete replacement of previously circulating RSV-A strains by the new genotype within just one year. This rapid diffusion of this strain was probably made possible by its genetic diversity in the G protein. Moreover, the ON1 study strains of the 2012/13 season displayed several amino acid substitutions compared with the ON1 prototype and with ON1 strains deposited in GenBank [18,23,30].

The variability and apparent evolution seen at the positively selected site 274 in the ON1 cluster of sequences is particularly interesting. All 2011/12 strains but one (12221AN) had a leucine in this position, like most

Table 3

Demographic and clinical data of respiratory syncytial virus-A-positive patients diagnosed during the 2011/12 and 2012/13 seasons, stratified by RSV-A genotype, Italy (n=99)

Features	RSV-A n=99[a]	Non-ON1 n=43	ON1 n=56	p value[b]
Median age, months (range)	2.8 (0.4–60)	5.0 (0.5–60)	2.5 (0.4–32)	**0.030**
Patient younger than one year, n (%)	85 (85.6)[c]	33 (76.7)	52 (92.8)	**0.039**
Male sex, n (%)	66 (66.6)	30 (69.8)	36 (64.3)	0.66
Clinical diagnosis[d]				
Bronchiolitis[e], n (%)	61 (61.9) {7}	26 (60.5) {1}	35 (62.5) {6}	0.838
Wheezing/asthma, n (%)	9 (9.1)	3 (7.0)	6 (10.7)	0.727
Bronchopneumonia, n (%)	16 (16.2) {4}	12 (27.9) {2}	4 (7.1) {2}	**0.011**
ILI[f], n (%)	13 (13.1) {1}	2 (4.6)	11 (19.6) {1}	**0.036**
PICU admission, n (%)	12 (12.1)	3 (7.0)	9 (16.1)	0.053
Bronchiolitis patients[g]	RSV-A n=52	Non-ON1 n=23	ON1 n=29	
Median age, months (range)	2.0 (0.3–9)	2.5 (0.3–8)	1.6 (0.5–9)	0.110
Male sex, n (%)	35 (67.3)	15 (65.2)	20 (69.0)	1.000
Respiratory rate >45 breaths/min, n (%)	35 (67.3)	19 (79.2)	16 (55.2)	**0.043**
O2 saturation <95%, n (%)	25 (47.2)	13 (54.2)	12 (41.4)	0.403
Retractions, n (%)	36 (69.2)	17 (73.9)	19 (65.5)	0.560
Severity score 4–8[h], n (%)	13 (25.0)	9 (39.1)	4 (13.8)	0.053

ILI: influenza-like-illness; PICU: paediatric intensive care unit; RSV: respiratory syncytial virus.

[a] Number of patients for whom data were available.

[b] Mann-Whitney test for the difference in median age; Fisher's exact test for analysis of independent categorical variables. Bold indicates a significant result.

[c] In parentheses the proportion of cases with the condition among the total cases per group.

[d] Clinical diagnosis on admission; number of PICU admissions in curly brackets.

[e] Bronchiolitis was defined as a history of upper respiratory tract infection followed by acute onset of respiratory distress with cough, tachypnoea, retraction and diffuse crackles on auscultation in infants within one year [20].

[f] ILI was defined according to the Italian Health Ministry as fever (temperature ≥38 °C), with at least one of the following symptoms: headache, asthenia, myalgia, and at least one of the following respiratory symptoms: cough, rhinitis, acute pharyngitis.

[g] Clinical data were available for 52 of 61 patients admitted for bronchiolitis (excluding PICU cases).

[h] Clinical severity score (0–8) including subscores for respiratory rate (<45 breaths/min=0, 45–60 breaths/min=1, >60 breaths/min=2); arterial oxygen saturation in room air (>95%=0, 95–90%=1, <90%=2); presence of retractions (none=0, present=1, present+nasal flare=2), and feeding ability (normal=0, reduced=1, intravenous=2) [20].

strains circulating then and in the previous winter as well as the ON1 prototype. The reversal mutation involving a proline at site 274, first detected in the 12221AN strain in the homologous portion and in the insert (LxvP), together with the YxxiH substitution, seemed to confer an evolutionary advantage, being found in most (31/52 sequences; 60%) 2012/13 strains. Notably, this variant was circulating during the 2011/12 winter as a minority strain also in Germany [30] and Japan (GenBank: AB698559). These variations may have arisen from independent evolutionary reversal events affecting the amino acid at site 274 and its homologous position in the insert, as may happen in those sites following the so called 'flip-flop' phylogenetic pattern (i.e. a frequently reversible amino acid replacement) [10]. Alternatively, the duplication event may have occurred independently in a strain already bearing the L274P substitution, generating the same substitution in its homologous position in the insert, followed by a second independent mutation at insert position xxi. ON1 strains with 274P and/or xvP in the homologous and insert portion spread widely in the population, accounting for 67% of 2012/13 strains.

Overall, a variety of genetic changes could be responsible for the spread of ON1 strains, conferring low cross-protection by pre-existing antibodies to RSV-A strains previously circulating in Italy: the 24 aa insertion, the loss of a further potential glycosylation site due to the T253K substitution, and even other amino acid changes. Unfortunately, we were not able to culture ON1 clinical isolates and could therefore not assess the antigenic properties of these strains.

Nonetheless, the ability to replace circulating RSV-A strains and the fact that the peak of RSV infections occurred earlier in 2012/13 than in the previous two seasons, could be the consequence of broad antigenic diversity. Another possible consequence of antigenic diversity could be that newborns infected with a novel genotype would be less protected by maternal antibodies against previously circulating RSV-A strains. This would be consistent with our finding that hospitalised patients with ON1 were younger than those with other RSV strains, as was also seen in a German report for July 2010 to June 2012 [30]. In contrast, a Japanese study reported that patients infected with a novel GA2 variant, NA2, had a greater mean age than those infected with the previously circulating GA5 genotype [7]. The difference could be related to viral characteristics or, more probably, to the study population, since the Japanese study described cases reported to the national surveillance by sentinel paediatric clinics, whereas all our samples were from hospitalised children; we could therefore not assess the impact of the novel genotype at the population level.

Besides the epidemiological impact, significant genetic variation in circulating strains may involve different pathogenicity and virulence. Several publications have recently documented that different patient isolates are able to induce variable pathogenesis in a mouse model [31] and in cell culture [32]. Moreover, novel substitutions and deletions were identified in RSV strains from clinical samples of severely ill patients [15].

In this study we analysed demographic and clinical data from about 100 RSV cases and from 52 well-characterised bronchiolitis patients, and found differences between infections with ON1 vs GA2 strains. ON1 patients were significantly younger than those infected with the other RSV-A strains (p=0.03); they were more frequently affected with less severe clinical conditions; bronchiolitis severity was lower, as documented by the significantly better respiratory condition (p=0.043) and the considerably lower severity score (p=0.053). A milder clinical course among children infected with genotype ON1 compared to infections with other RSV infections was recently reported in Cyprus [33]. On the other hand, PICU admissions were more numerous for ON1 than for the other RSV-A strains (p=0.053), as also reported in the German study [30]. Given these contrasting observations, the novel genotype does not seem to possess special determinants of severity compared with previously circulating RSV-A strains, but its sudden diffusion due to genetic differences could have increased the epidemic peak, the number of hospitalised patients and consequently PICU admissions.

Undoubtedly, it is of interest to investigate viral and host pathogenic factors during both severe and mild RSV infection, but the challenge is to characterise RSV strains and clinical conditions from a sufficiently large number of patients to assess associations. In several European countries, weekly reports of influenza virus characterisation also contain RSV detection in ILI cases reported by sentinel primary care physicians, issued by the European Influenza Surveillance Network (EISN). In Italy, RSV testing of ILI samples was performed in the framework of the EISN RSV Task Group up to year 2007 [34].

The Task Group's report recommended improving RSV surveillance using molecular techniques yet to be standardised and setting up a sentinel system of representative hospitals to determine the burden of RSV illness and define its epidemiology [34]. Research projects would now be well timed to monitor the diffusion of the novel ON1 genotype and of the other RSV strains in the general population and to determine their hospitalisation rate and clinical impact. This knowledge could also help include the more virulent strains in vaccines.

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Conflict of interest

None declared.

Authors' contributions

AP and PB designed the study, had primary responsibility for analysis of the results and wrote the paper; DT conducted research in Ancona and performed phylogenetic analysis; CS conducted research in Rome; MLF and KM performed virological diagnosis and collected patient data in Ancona; AN and FM collected respiratory samples and patient data in Rome; GA analysed the results and contributed to writing the paper.

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