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High epidemic burden of RSV disease coinciding with genetic alterations causing amino acid substitutions in the RSV G-protein during the 2016/2017 season in The Netherlands

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ABSTRACT

Background: We found amino acid substitutions in the Gglycoprotein of respiratory syncytial virus (RSV) A during the 2016/2017 epidemic in The Netherlands.
Objectives: We evaluated whether these alterations led to increased RSV incidence and disease burden.
Study design: We sequenced the gene encoding the G-protein of prospectively collected clinical specimens from secondary care adult patients testing positive for RSV during the 2016/2017 and 2017/2018 epidemic RSV season. We evaluated associations between genetic, clinical and epidemiological data.
Results: We included 49 RSV strains. In 2016/2017 28 strains were included, 20 community acquired RSV-A, 5 hospital acquired RSV-A and 3 community acquired RSV-B. In 2017/2018 21 strains were included, 8 community acquired RSV-A and 13 community acquired RSV-B. G-proteins of 10 out of the 20 community acquired 2016/2017 RSV-A strains shared a set of eight novel amino acid substitutions of which seven in mucin-like regions 1 and 2 and one in the heparin binding domain. This genetic variant was no longer detected among 2017/2018 RSV-A strains. Among patients carrying the novel RSV-A strain-type, 30% died.
Conclusions: A set of eight amino acid substitutions was found in 50% of the 2016/2017 community acquired RSV-A G-proteins. This combination of substitutions was globally never observed before. The appearance of this new strain-type coincided with an increased RSV peak in The Netherlands and was associated with higher disease severity. The transient character of this epidemic strain-type suggests rapid clearance of this lineage in our study community.

1. Background

Human respiratory syncytial virus (RSV) is an important cause of respiratory tract infections (RTIs). It is a single-stranded, negative-sense, enveloped RNA virus from the *Pneumoviridae* family [1]. The virus is most harmful in neonates, children with chronic lung or congenital heart diseases, with 1.9% mortality among hospitalized children ≤5 years who present with RSV associated acute lower RTIs [2] and immunocompromised or elderly patients [3] with 8.0% mortality in hospitalized RSV positive adults ≥65 years [3]. RSV has two subtypes, RSV-A and RSV-B, which is based on antibody interactions with the two major glycoproteins on the surface of the RSV virion that initiate infection [4], the F (fusion) and G (attachment) proteins [5]. The F-protein, which is present and typical for all *Pneumoviridae,* directs viral penetration and syncytium formation (fusion) [4]. The G-protein, which is unique in RSV compared to other *Pneumoviridae*, is involved in attachment to the ciliated cells of the respiratory tract [4]. The genome of RSV is about 15,200 nucleotides in length [5]. RSV, like other RNA viruses, is highly genetically variable. Over the last two decades, mutations in the gene encoding the G-protein accounted for the largest proportion of genetic variation within RSV, which potentially affects the ability of the virus to attach to cells of the respiratory tract [6]. RSV-A currently has at least eleven genotypes: GA1 to GA7, SAA1, NA1, NA2 and the newest genotype ON1, the latter containing a 72 nucleotide

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duplication in the region encoding the C-terminus of the G-protein [6]. During an epidemic RSV season typically more than one genotype from the same RSV subgroup co-circulates within a community with a clear predominant genotype every season that shifts over time [7]. In a study among infants with RSV bronchiolitis in the USA, the predominant genotype shifted from GA5 (2004–2006) to GA2 (2007–2010) to the new ON1 genotype during the season 2010-2011[8]. In 2013, GA2 was reintroduced as dominant RSV-A genotype in the USA [7], but in other countries like China ON1 remained predominant until 2015 [9]. RSV-B currently has at least 17 genotypes: GB1 to GB4, SAB1 to SAB3 and BA1 to BA10 [10]. During the last decade, the BA genotypes circulate at similar rates [11], accounting for around 70% of RSV-B infections [7,8].

Information on the genetic evolution of RSV may help explain variations between seasons in disease severity [12], although the association between genetic alterations and virulence is disputed [8,13]. Furthermore, insight in genetic evolution of RSV may provide targets for replication inhibitors [14] and preventive RSV vaccines which are currently widely being developed [15]. Finally, genetic alterations in the virus could help us understand seasonal variability of RSV incidence, which may of course also be influenced by other factors as meteorology [16] and the occurrence of other respiratory viruses like influenza virus [17].

2. Objectives

In this study we therefore provide a description of the G-protein of RSV present in clinical specimens from adult patients during two consecutive seasons. In addition, we evaluate whether there is an association between genetic changes and the disease burden of RSV.

3. Study design

3.1. Patient selection

From 198 RSV positive adult patients from two hospitals, tested during two consecutive epidemic RSV seasons, a random sample was taken. Both the University Medical Center Utrecht (UMCU) and Academic Medical Center (AMC) in Amsterdam are large tertiary care hospitals with over 1000 hospital beds each, located in urbanized regions in the center of The Netherlands. The epidemic RSV seasons were defined by the national surveillance Center (RIVM) and lasted from November 7th 2016 till March 12th 2017 and November 13th 2017 till April 8th 2018, respectively [18]. In the UMCU patients were included when they presented at the Emergency Department (ED) with symptoms or a working diagnosis of an RTI, regardless of whether they were admitted or not (611 patients in the 2016/2017 season, 932 patients in the 2017/2018 season). Respiratory nasopharyngeal samples were collected as part of routine care within 24 h of admission. From the AMC only samples collected during the first season were included from patients developing RTI symptoms during hospitalization. RSV was initially confirmed in all patients using multiplex RT-PCR on respiratory nasopharyngeal samples. RSV detections from samples collected at the ED or within 48 h of admission, were defined as community acquired RSV, whereas others were defined as hospital acquired RSV. This study obtained ethical approval from the local ethics committee of the UMCU, protocol number 16-692/C.

3.2. Clinical data collection

Clinical data were collected manually from the Electronic Medical Records. Immunocompromised status was defined as use of corticosteroids or other immunosuppressive drugs with a cumulative dose of > 700 mg prednisone or equivalent, stem cell transplantation, organ transplantation, use of biologicals of anti-rheumatics during the last 6 months and/or asplenia, primary immunodeficiency and/or controlled HIV-infection with CD4-penia < 200/mm3. The CURB-65 score, which reflects disease severity, was calculated by counting one point for every item that was present: (1) confusion, (2) urea > 7 mmol/L, (3) respiratory rate ≥30 per minute, (4) systolic blood pressure < 90 mmHg or diastolic blood pressure ≤60 mmHg, (5) age ≥65 years.

Real-time Taqman RT-PCR RSV detection, nucleic acid isolation and G-gene sequencing - Details are described in Supplementary Text 1 [19,20].

3.3. Data analysis

The complete alignment of the G-genes of all RSV-A positive clinical specimens included during the 2016/2017 and 2017/2018 seasons were compared to local RSV-A sequences that were collected during 15 previous seasons, as well as to global G-gene sequences previously grouped and analyzed by Zou et al. [21]. Nucleotide and protein aliment was performed with MegAlign volume 15 (DNASTAR Lasergene) using Clustal W method. Statistical analysis was performed using Excel (Microsoft Office, Excel 2010) and SPSS (IBM SPSS Statistics 21). Missing clinical categorical variables were imputed using multiple imputation. For continuous data, complete case analysis was performed. Descriptive data were given in percentages for categorical data or median with interquartile range (IQR) for continuous data. To exclude bias when evaluating the genetic evolution of RSV, we excluded the small group (n = 5) of patients with hospital acquired RSV. To compare groups, Pearson chi-square or Fisher's exact tests were used for categorical data, a one-way ANOVA with post-hoc Fisher LSD test for continuous data when comparing three groups and Mann Whitney *U* test for continuous data when comparing two groups. Statistical significance was set at $p < 0.05$.

4. Results

Forty-nine patients were included (median age 59.9 years [IQR 42.9–67.6]). During the 2016/2017 respiratory season, 28 patients were included among which 20 with community acquired RSV-A (19 UMCU; 1 AMC), 5 with hospital acquired RSV-A (AMC) and 3 with community acquired RSV-B (UMCU). During the 2017/2018 respiratory season, 21 patients were included (UMCU), among which 8 patients with community acquired RSV-A and 13 with community acquired RSV-B. Complete amino acid alignments of the G-protein of all included patients are shown in *Supplementary* Fig. 1. Ten out of 20 patients with community acquired RSV-A from the 2016/2017 season had strains characterized by the presence of eight amino acid substitutions previously never observed in that combination. All but one of the substitutions were found in mucin-like regions 1 (MLR-1; 67H-164 H) and 2 (MLR-2; 224E-321 K), K216 N occurred within the heparin binding domain (HBD; 186C-224E) (Fig. 1). Among the eight substitutions were three previously largely conserved amino-acids, i.e. S102 F, P256S and H258Y. A second circulating set of mutations was found in seven other 2016/2017 RSV-A strains. Five of these RSV-A strains were hospital-acquired, obtained from patients hospitalized in the same center (AMC) during a short time frame and were therefore excluded from further genetic analyses. In 11/16 RSV-B strains one substitution (T288I) was added to the repetitive set of mutations (T107 A; R136 T; X198 T; T152I; I179 T) that was seen within our local RSV-B strains since 2014.

The complete alignment of the G-protein of RSV-A of all community acquired strains included during the 2016/2017 and 2017/2018 seasons was compared to local RSV-A strains collected during the 15 previous seasons. In line with previous reports [7], major changes of the RSV-A G-protein at the level of amino acids were observed periodically every 5–6 seasons. Clearly, we observed clustering between strains collected between 2001 and 2006 (genotype GA5), followed by a cluster of strains collected between 2006 and 2011 (GA2) and finally the strains collected from 2012 till 2017 showing a 72bp repeat (ON1). When comparing our community acquired RSV-A sequence data to

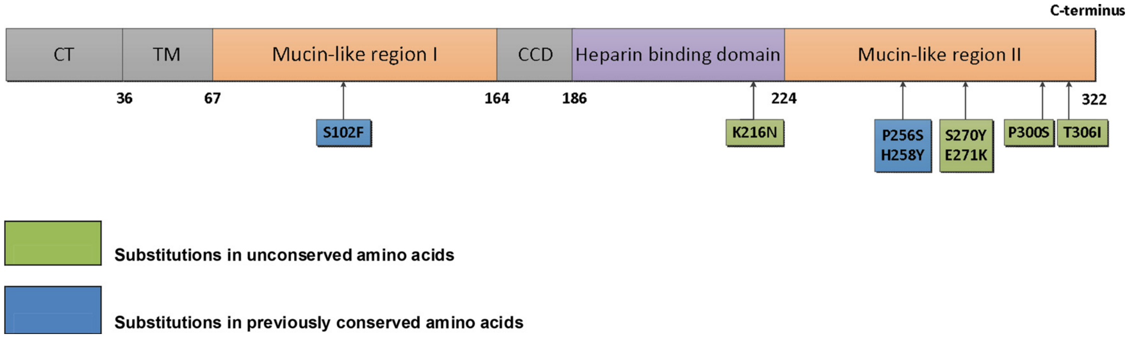


Fig. 1. Fixed set of substitutions found in the G-glycoprotein of RSV-A (10/20 community acquired RSV-A positive patients during the 2016–2017 epidemic RSV season).

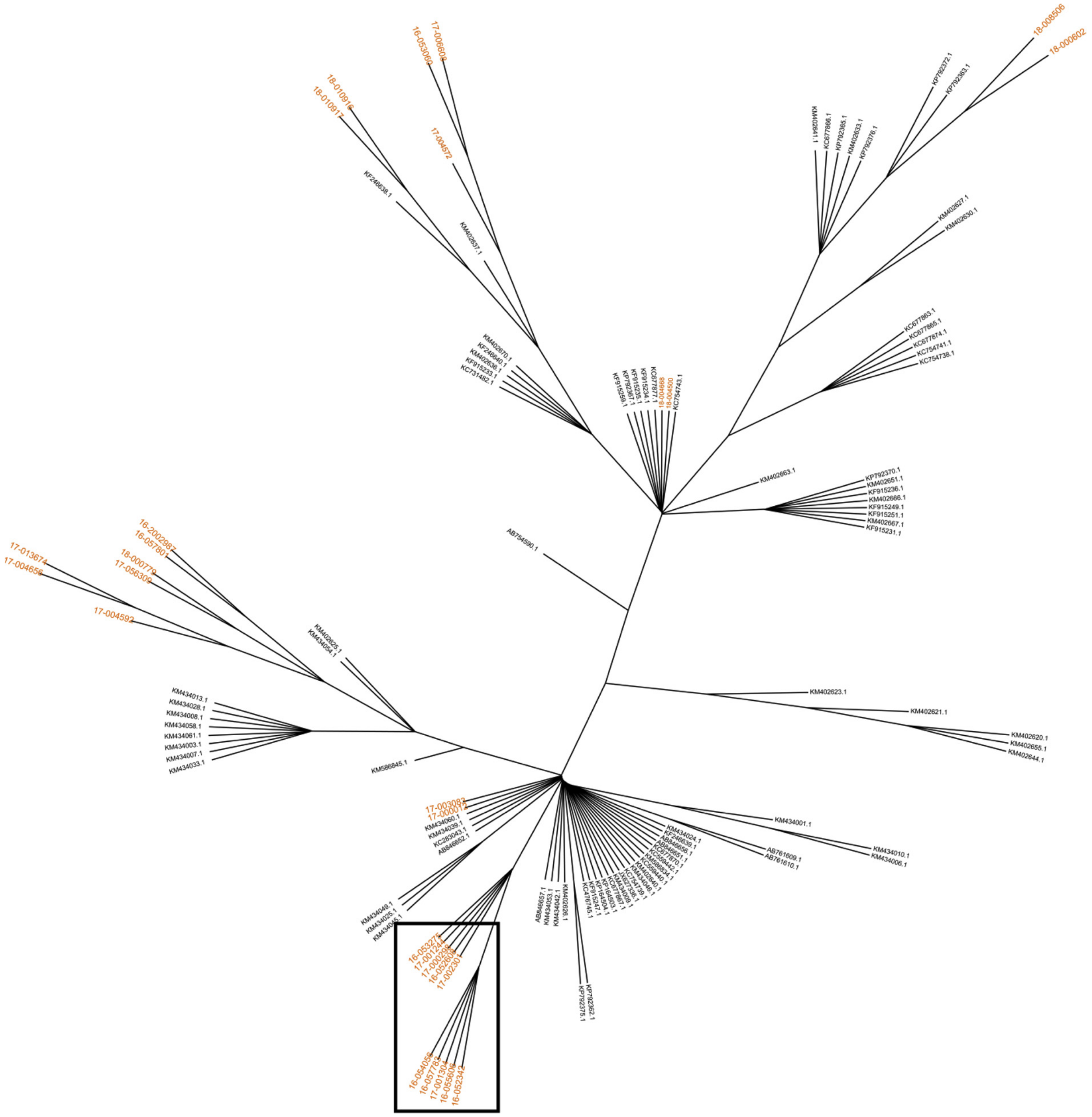
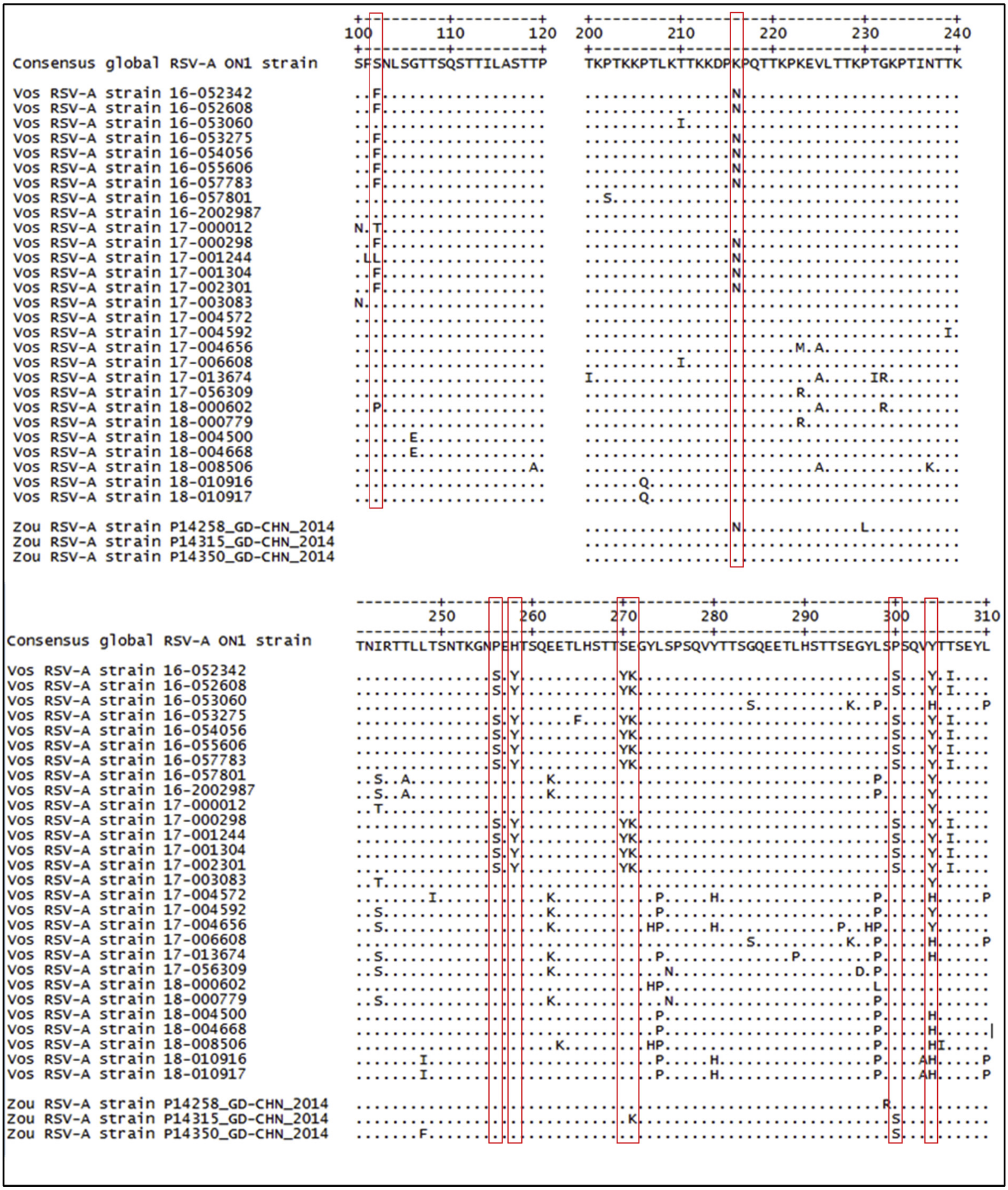


Fig. 2. Circular phylogenetic tree of global ON1 G-gene sequences. In orange we depicted community acquired RSV-A sequences form the 2016/2017 and 2017/2018 season (n = 28; this paper). Boxed the 10 RSV-A strains with the set of 8 amino acid substitutions (2016/2017 season), that clearly cluster together. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



```
                                     +---------+---------+   +---------+---------+---------+---------+
                                     100       110     120   200       210       220       230     240
                                     +---------+---------+   +---------+---------+---------+---------+
Consensus global RSV-A ON1 strain    SFSNLSGTTSQSTTILASTTP   TKPTKKPTLKTTKKDPKPQTTKPKEVLTTKPTGKPTINTTK

Vos RSV-A strain 16-052342           ..F..................   ................N........................
Vos RSV-A strain 16-052608           ..F..................   ................N........................
Vos RSV-A strain 16-053060           .....................   ..........I..............................
Vos RSV-A strain 16-053275           ..F..................   ................N........................
Vos RSV-A strain 16-054056           ..F..................   ................N........................
Vos RSV-A strain 16-055606           ..F..................   ................N........................
Vos RSV-A strain 16-057783           ..F..................   ................N........................
Vos RSV-A strain 16-057801           .....................   ..S......................................
Vos RSV-A strain 16-2002987          .....................   .........................................
Vos RSV-A strain 17-000012           N.T..................   .........................................
Vos RSV-A strain 17-000298           ..F..................   ................N........................
Vos RSV-A strain 17-001244           .LL..................   ................N........................
Vos RSV-A strain 17-001304           ..F..................   ................N........................
Vos RSV-A strain 17-002301           ..F..................   ................N........................
Vos RSV-A strain 17-003083           N....................   .........................................
Vos RSV-A strain 17-004572           .....................   .........................................
Vos RSV-A strain 17-004592           .....................   .......................................I.
Vos RSV-A strain 17-004656           .....................   .......................M.A...............
Vos RSV-A strain 17-006608           .....................   ..........I..............................
Vos RSV-A strain 17-013674           .....................   I........................A.....IR........
Vos RSV-A strain 17-056309           .....................   .......................R.................
Vos RSV-A strain 18-000602           ..P..................   .........................A......R........
Vos RSV-A strain 18-000779           .....................   .......................R.................
Vos RSV-A strain 18-004500           ......E..............   .........................................
Vos RSV-A strain 18-004668           ......E..............   .........................................
Vos RSV-A strain 18-008506           ...................A.   .........................A...........K...
Vos RSV-A strain 18-010916           .....................   ......Q..................................
Vos RSV-A strain 18-010917           .....................   ......Q..................................

Zou RSV-A strain P14258_GD-CHN_2014                          ................N.............L..........
Zou RSV-A strain P14315_GD-CHN_2014                          .........................................
Zou RSV-A strain P14350_GD-CHN_2014                          .........................................


                                     ---------+---------+---------+---------+---------+---------+---------+
                                             250       260       270       280       290       300       310
                                     ---------+---------+---------+---------+---------+---------+---------+
Consensus global RSV-A ON1 strain    TNIRTTLLTSNTKGNPEHTSQEETLHSTTSEGYLSPSQVYTTSGQEETLHSTTSEGYLSPSQVYTTSEYL

Vos RSV-A strain 16-052342           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 16-052608           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 16-053060           ...........................................S..........K..P.....H.....P
Vos RSV-A strain 16-053275           ...............S.Y......F....YK............................S...Y.I....
Vos RSV-A strain 16-054056           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 16-055606           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 16-057783           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 16-057801           ..S..A...............K...................................P.....Y......
Vos RSV-A strain 16-2002987          ..S..A...............K...................................P.....Y......
Vos RSV-A strain 17-000012           ..T............................................................Y......
Vos RSV-A strain 17-000298           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 17-001244           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 17-001304           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 17-002301           ...............S.Y...........YK............................S...Y.I....
Vos RSV-A strain 17-003083           ..T............................................................Y......
Vos RSV-A strain 17-004572           ........I............K...........P.....H.................P.....H.....P
Vos RSV-A strain 17-004592           ..S..................K...........P.......................P.....Y......
Vos RSV-A strain 17-004656           ..S..................K..........HP.....H.............P..HP.....Y......
Vos RSV-A strain 17-006608           ...........................................S..........K..P.....H.....P
Vos RSV-A strain 17-013674           ..S..................K...........P..............P........P.....H......
Vos RSV-A strain 17-056309           ..S..................K............N....................D.P............
Vos RSV-A strain 18-000602           ................................HP.......................L............
Vos RSV-A strain 18-000779           ..S..................K............N......................P............
Vos RSV-A strain 18-004500           .................................P.......................P.....H......
Vos RSV-A strain 18-004668           .................................P.......................P.....H......|
Vos RSV-A strain 18-008506           ......................K.........HP.......................P.....HI.....
Vos RSV-A strain 18-010916           .......I.........................P.....H.................P....AH.....P
Vos RSV-A strain 18-010917           .......I.........................P.....H.................P....AH.....P

Zou RSV-A strain P14258_GD-CHN_2014  ..........................................................R...........
Zou RSV-A strain P14315_GD-CHN_2014  ..............................K............................S..........
Zou RSV-A strain P14350_GD-CHN_2014  .......F...................................................S..........
```

Fig. 3. G-protein sequences alignment of ON1 genotype RSV-A. The regions in the C-terminus of G-protein sequences in which the mutations from the set of 8 mutations were found, are shown (amino acids 100–120; 200–310). Red boxes depict the 8 found mutations. The consensus ON1 strain (upper line) was based on global ON1 strains with exclusion of strains from this paper. The 28 community acquired RSV-A strains described in the current study are depicted as 'Vos RSV-A strain', in chronological order. At the bottom we added three strains published by Zou et al (2016) in which at least one of the mutations of interest was found; amino acids 100–120 are not available for these strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

global RSV-A sequences, we found that our strains cluster randomly within the ON1 genotype (Fig. 2). The ten strains displaying the set of 8 amino acid substitutions, however, clearly form a separate branch in the ON1 cluster. When investigating the occurrence of the 8 amino acid substitutions in global ON1 strains (n = 102), only the K216 N (n = 6), E271 K (n = 3) and P300S (n = 4) substitutions were found. Only two strains showed a combination of the E271 K and P300S substitutions. The majority of these mutations (n = 9) was found in local Guangdong 2014 & 2015 ON1 strains [21] (Fig. 3).

Clinical characteristics of all 44 included adult patients with community acquired RSV are given in Table 1. 31 patients (70.5%) were immunocompromised and 18 (40.9%) had a working diagnosis of pneumonia based on radiological findings. Twenty-seven patients (61%) were admitted, of whom four died during their hospital stay with

Table 1

Baseline characteristics of patients with PCR proven community acquired RSV included in this study (n = 44), divided by community acquired RSV A positive patients with the new cluster of mutations (n = 10), other community acquired RSV A positive patients (n = 18) and community acquired RSV B positive patients (n = 16).

Characteristics	RSV A mutated (n = 10)	RSV A non-mutated (n = 18)	RSV B (n = 16)	p-value*
Age (years) – median (IQR)	67.0 (37.4–74.3)	53.3 (25.1–62.6)*	63.1 (58.8–68.8)*	0.048
Male sex - no. (%)	5 (50%)	10 (56%)	7 (44%)	0.791
(Ex-)smoker - no. (%)	4 (40%)	9 (50%)	7 (44%)	0.861
Comorbidities				
Immunocompromised - no. (%)	6 (60%)	13 (72%)	12 (75%)	0.765
Diabetes - no. (%)	4 (40%)	3 (17%)	2 (13%)	0.275
Asthma or COPD - no. (%)	3 (30%)	3 (17%)	4 (25%)	0.729
Chronic renal failure or hearth failure - no. (%)	7 (70%)*	4 (22%)*	5 (31%)	0.043
Current hematologic malignancy - no. (%)	2 (20%)	5 (28%)	1 (6%)	0.320
Diagnostic findings at presentation				
Symptom duration (days) - median (IQR)	3.0 (2.0-6.3)	3.0 (1.0-5.5)	4.5 (2.3-7.0)	0.731
Symptoms at presentation:	10 (100%)	17 (94%)	14 (88%)	0.598
Cough - no. (%)	10 (100%)	17 (94%)	14 (88%)	0.598
Dyspnoea - no. (%)	10 (100%)	12 (67%)	15 (94%)	0.042
Delirium - no. (%)	3 (30%)	3 (17%)	1 (6%)	0.337
Temperature (^{0}C) - median (IQR)	37.9 (36.7–38.6)	38.2 (37.7–38.5)	37.4 (36.5–38.3)	0.252
Fever (temperature $\geq 38.0^{0}$C) - no. (%)	4 (40%)	10 (56%)	5 (31%)	0.312
CRP (mg/L) - median (IQR)	68.5 (18.3–171.0)*	24.5 (11.3–58.2)*	24.0 (11.8–56.3)	0.126
Leukocytes (x10ˆ9/L) - median (IQR)	9.1 (6.1–11.7)	9.3 (5.3–12.1)	8.7 (6.1–10.9)	0.813
Disease severity and clinical outcomes				
PaO2 (mmHg) - median (IQR)	61.0 (51.5–74.5)	68.0 (59.0–78.0)	66.0 (60.0–72.0)	0.436
O2-suppletion needed ($\geq$1 L) - no. (%)	8 (80%)*	5 (28%)*	10 (63%)	0.020
CURB-65 score** - median (IQR)	1.5 (0.8–3.0)*	0.0 (0.0–2.0)	0.5 (0.0–1.0)*	0.079
Diagnosis RTI at presentation - no. (%)	8 (80%)	14 (78%)	15 (94%)	0.439
Pneumonia based on X-ray - no. (%)	6 (60%)	7 (39%)	5 (31%)	0.419
Length of hospital stay (days) - median (IQR)	3.5 (1.5–8.8)	0.0 (0.0–9.5)	6.0 (0.0–11.5)	0.933
In-hospital death - no. (%)	3 (30%)	1 (6%)	0 (0%)	0.045
Treatment started at presentation				
Hospital admission - no. (%)	8 (80%)	8 (44%)	11 (69%)	0.155
Directly admitted to ICU or MCU - no. (%)	1 (10%)	3 (17%)	2 (13%)	1.000
Aerogenic isolation measurements - no. (%)	4 (40%)	5 (28%)	7 (44%)	0.665
Antibiotics - no. (%)	6 (60%)	13 (72%)	8 (50%)	0.453
Oseltamivir - no. (%)	4 (40%)	7 (39%)	4 (25%)	0.713

COPD, Chronic Obstructive Pulmonary Disease; CRP, C-reactive protein; CURB-65, confusion, urea, respiratory rate, blood pressure, age > 65 years; ED, emergency department; MCU, Medium Care Unit; ICU, Intensive Care Unit; IQR, interquartile range; no., number; O2, oxygen; pO2, partial pressure of oxygen measured in arterial blood sample; RTI, respiratory tract infection.

Substituted RSV-A, non-substituted RSV-A and RSV-B positive patients with community acquired RSV infection (n = 44) were compared by performing an one-way ANOVA test for continuous variables and a chi square test or Fisher's exact test for categorical variables. In case of overall significance, a post-hoc analysis was done using Fisher LSD for continuous variables and using chi-square test for two groups for categorical variables. Significant differences between two groups as assessed by post-hoc test is indicated with *. ** CURB-65 score consists of 5 items. 1 point is given per positive item: confusion, urea > 7 mmol/L, respiratory rate ≥30 per minute, systolic blood pressure < 90 mmHg or diastolic blood pressure ≤60 mmHg and age ≥65 years.

a median time between hospital admission and death of 23 days [IQR 3.5–61 days]. The median age of the four patients who died was 72.2 years [IQR 63.4–74.3], two were immunocompromised. In univariate analysis, RSV-A positive patients with the presence of 8 substituted amino acids (n = 10) more often were dyspneic (10% vs 67–94%, p = 0.042) as compared to patients with community acquired RSV-A without the 8 mutated amino acids (n = 18) and RSV-B positive patients (n = 16). Furthermore, RSV-A positive patients with the 8 substituted amino acids seemed to have a higher disease severity with more frequent need for extra oxygen (80% vs 28–63%, p = 0.020) and a higher in-hospital mortality rate (30% vs 0–6%, p = 0.045). Also, as compared to patients with RSV-B, RSV-A positive patients with the 8 substituted amino acids had a higher median CURB-65 score (1.5 [IQR 0.8–3.0] vs 0.5 [IQR 0.0–1.0], p = 0.029). After adjustment for differences in age and renal or congestive heart failure, patients with RSV-A with the 8 substituted amino acids had 3.8 fold (95% confidence interval, 0.3–57.3) higher odds for in-hospital mortality as compared to other RSV-A patients.

5. Discussion

During the 2016/2017 epidemic RSV season, we found an RSV-A strain characterized by the presence of 8 substituted amino acids within the G-protein previously never observed in that combination in 50% of our adult patients with community acquired RSV-A. Within this set of substitutions are three previously largely conserved amino-acids within MLR-1 and MLR-2 (S102 F, P256S and H258Y) and one (K216 N) within the HBD (186T-224E).

Here, we focused on the G-protein, as we have previously shown that the gene encoding this protein shows the highest genetic variability [19] and is able to correlate a set of mutations to a disease phenotype. Among the 10 patients with the 8 substituted amino acids, three patients died during their hospitalization due to RSV, while none of the RSV-B positive patients (n = 16) and only one of the other RSV-A positive patients (n = 18) died (p = 0.045). Furthermore, disease severity as reflected by the CURB-65 score, was higher in RSV-A positive patients with the substitutions as compared to RSV-B positives (p = 0.029). These differences might have been affected by potential confounders as age and chronic renal and/or congestive heart failure, which differed at baseline. However, from these small numbers with few events it is difficult to draw conclusions after multivariate adjustment. In addition, other potentially confounding factors as host genetics and immunity induced by genotypes previously circulating in a community were not addressed in this study.

Although it is tempting to hypothesize on the existence of a causative relationship between the observed viral G-protein changes and the burden of disease, those additional factors should be studied thoroughly in follow-up structure-function relating studies. We plan to study the impact of X216 N, a substitution that yields an extra N-glycosylation site. N-glycosylation is often involved in modification of protein structure and stability [22], as well as modulation of host cell-surface interactions [23], enzyme activity [24] and hiding of antigenic sites, allowing the virus to escape neutralizing antibodies [22,25]. For most proteins, optimal N-glycosylation is considered to be important for optimization of the intrinsic dynamic properties of a certain protein [26]. N-glycosylation is only predicted at seven positions [19] in this protein, none of which being located within the conserved HBD. Hence we will analyze replication capacity in one-step growth curves, as well as antibody escape in neutralization experiments.

The suggestion that this set of mutations might precede a new genotype within RSV-A is attenuated by the fact that the set of mutations was not seen again during the subsequent season of 2017/2018 in which RSV-B dominated [18]. Interestingly, though speculative, an explanation for the immediate extinction of the 2016/2017 lineage could very well represent an increase in antigenicity of this lineage, resulting in a strong reduction of susceptible hosts in the subsequent winter season as was seen before in other *Paramyxoviridae* as measles [27]. Therefore, we plan to analyze the behavior of the genetically altered strains in neutralization experiments.

The second notable finding is that the genomic changes in RSV-A not only seem associated with higher disease severity, but also coincide with increased RSV incidence. During the 2016/2017 epidemic RSV season, there was a higher peak in RSV incidence as compared to the previous five years and compared to the 2017/2018 season [18] (*Supplementary* Fig. 2). Since the mutated RSV-A strains we found were collected on average in week 51 of 2016 (standard deviation 4.0 weeks), right at the middle of the peak, the mutated RSV-A lineage might have contributed to this increased RSV incidence.

The major limitation of the current study is the small amount of samples. Although the predominance of RSV-A during the 2016/2017 season and predominance of RSV-B during the 2017/2018 season reflects the national trends, the number of RSV-A positive strains that could be analyzed during the second season was limited. However, since the RSV-A positive patients included during the second season were randomly selected and all had community acquired RSV-A, we can safely conclude that the circulation of this strain type is at least strongly reduced. Even though the current study might have limited power, the combination of clinical data and high-quality genetic data of RSV positive adult patients from two consecutive seasons is valuable.

In conclusion, the current study showed a transient change in the gene encoding the G-protein of community acquired RSV-A during the 2016/2017 winter, associated with increased disease severity and RSV incidence in a small group of patients. Interestingly, the eight amino acid residue substitutions included one within the heparin binding domain, which might yield an extra N-glycosylation site and give rise to increased virulence. Furthermore, the location of our RSV-A strains within the ON1 genotype and the occurrence of 3 out of 8 mutations in Chinese ON1-strains, confirm the current hypothesis that local RSV-A epidemics are caused by a combination of viruses seeded from external regions and persistence of local viruses [21]. Maintenance of this lineage in other parts of the world, or a come-back in our region remains to be monitored.

Conflicts of interest

Prof. dr. Bont has regular interaction with pharmaceutical and other industrial partners (AbbVie, MedImmune, Janssen, the Bill and Melinda Gates Foundation, MeMed Diagnostics, Regeneron, Ablynx, Bavaria Nordic, MabXience and Novavax). He has not received personal fees or other personal benefits. All other authors have no commercial or other association that might pose a conflict of interest.

Credit author statement

LV and FC contributed to conceptualization and writing of the manuscript. LV and SK collected clinical data of included patients from the UMC Utrecht and AMC Amsterdam, respectively. AH, LB and JJO contributed to clinical supervision. MV performed sequencing of all included RSV strains. LV performed all formal analyses. All authors contributed to reviewing and editing of the manuscript.

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Ethical approval

This study obtained ethical approval from the local ethics committee of the University Medical Center Utrecht, protocol number 16-692/C.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.01.007.

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