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Research paper

Molecular epidemiology of human respiratory syncytial virus and human metapneumovirus in hospitalized children with acute respiratory infections in Croatia, 2014–2017

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ABSTRACT

Acute respiratory infection (ARI) is the most common infection in children under 5 years of age and it is frequently caused by two pneumoviruses, human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV). Epidemic seasons of these viruses overlap and disease manifestations are highly similar, including severe lower ARI such as bronchiolitis or pneumonia. Reinfections with pneumoviruses are frequent and limited prevention treatment is available. Genetic diversity of HRSV and HMPV strains circulating in Croatia was monitored during four consecutive years (2014–2017). Co-circulation of multiple lineages was observed for both viruses. Within HRSV group A, ON1 strains gained strong predominance during the 4-year period, while previously dominant genotype NA1 was detected only sporadically. Similarly, newly occurring HMPV genotype A2c gained predominance over genotype A2b during this period, resulting in all infection in 2017 being caused by A2c. Along with phylogenetic analysis based on the commonly used fragments for detection and genotyping of these viruses, full length G and SH genes were also analysed. Evolutionary dynamics showed that inferred substitution rates of HRSV and HMPV are between 2.51×10^{-3} and 3.61×10^{-3} substitutions/site/year. This study established presence of recently described HMPV strains containing large duplications in the G gene in Croatia. Viruses with either of the two duplications belong to a subcluster A2c, which has completely replaced all other group A subclusters in 2017.

1. Introduction

Human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV), both members of the *Pneumoviridae* family (Amarasinghe et al., 2017), account for the majority of lower respiratory tract infections worldwide in very young children, immunocompromised patients and less often the elderly. The disease manifestation following the infection is quite similar, ranging from mild nonspecific respiratory symptoms to severe illness, such as bronchiolitis or pneumonia (Collins and Karron, 2013). Furthermore, occurrence of HRSV and HMPV epidemics overlap, with HMPV peaks observed slightly later than HRSV peaks (Schildgen et al., 2011).

Pneumoviruses possess a negative-sense, single-stranded non-segmented RNA genome and express 3 surface proteins: the F (fusion glycoprotein), G (attachment protein) and SH (small hydrophobic protein). G and F proteins promote attachment and fusion with the target cell, respectively (Collins and Karron, 2013), however it was shown that G protein of both viruses is dispensable for *in vitro* replication (Biacchesi et al., 2004; Karron et al., 1997) suggesting that F protein also plays a major role in viral attachment (Cox et al., 2012; Mastrangelo and Hegele, 2013). In contrast to highly conserved F protein, G protein is extremely divergent and changes in this protein have been related to immune evasion, partly explaining frequent reinfections with pneumoviruses (Papenburg and Boivin, 2010). The third surface protein, SH is the most diverse genomic region next to G protein (Biacchesi et al., 2003; Schobel et al., 2016). SH protein is dispensable for replication *in vitro* (Biacchesi et al., 2004; Bukreyev et al., 1997), although it was shown that HRSV lacking SH is attenuated *in vivo*

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(Whitehead et al., 1999). Such effect was not observed for SH of HMPV (Biacchesi et al., 2005). SH protein may serve as a viroporin (Gan et al., 2012; Masante et al., 2014), and its role has been linked to evasion of innate immune responses, but the exact mechanism has not been fully elucidated (Fuentes et al., 2007; Hastings et al., 2016).

HRSV and HMPV are each classified into two broad groups, A and B, based on antigenic variation and sequence analysis (Boivin et al., 2002; Mufson et al., 1985; van den Hoogen et al., 2002). Both viruses show a rather complex circulation pattern, as numerous genotypes/subgroups within each group have been identified and co-circulation of genotypes within the same community is common (Papenburg and Boivin, 2010). Furthermore, new genotypes emerge and quickly spread worldwide replacing previously circulating genotypes (Trento et al., 2010). Molecular analysis of HRSV is based on the G gene sequences, particularly the second hypervariable region (HVR2) (Johnson et al., 1987; Peret et al., 1998). In addition to high variability of this region accounting for numerous genotypes within both groups, it was shown that it can accommodate large duplications: a 60-nt in-frame duplication in HVR2 led to the emergence of the BA genotype within group B (Trento, 2003) and a 72-nt in-frame duplication in HVR2 led to the emergence of genotype ON1 in group A (Eshaghi et al., 2012). Viruses with these duplications quickly spread globally and show strong epidemiological prevalence (Kim et al., 2014; Pierangeli et al., 2014; Trento et al., 2010).

Based on partial F gene sequences, HMPV group A consists of two subgroups A1 and A2, and group B is divided into B1 and B2 (Huck et al., 2006). Additionally, subgroup A2 can be further divided into subclusters A2a and A2b (Biacchesi et al., 2003; Huck et al., 2006). More recently, findings from Japanese (Nidaira et al., 2012) and Malaysian (Chow et al., 2016) epidemiological studies revealed an additional A2 subcluster, named A2c by Nidaira et al. (2012). We have demonstrated occurrence of this genotype in Europe (Jagušić et al., 2017). Recently it was found that the HMPV G protein follows a similar evolutionary pattern as was observed for HRSV. Two large duplications in group A, either 111 or 180 nucleotides long, were reported by Japanese (Saikusa et al., 2017a; Saikusa et al., 2017b) and Spanish authors (Piñana et al., 2017). Even though both duplications happened within the same region of the G gene, they were produced by independent duplication events (Saikusa et al., 2017b).

Due to the lack of licensed vaccines for either of the viruses, continuous molecular and epidemiological surveillance is recommended for better understanding of these diverse and fast evolving viruses. Therefore, the aim of this study was to investigate the diversity of HRSV and HMPV strains which produced acute respiratory infections (ARI) in Croatian children during four consecutive seasons (2014–2017). Along with phylogenetic analysis based on the commonly used fragments for detection and genotyping of these viruses, our study included full length G and SH genes, since these are the most diverse genomic regions. Selective pressure, evolutionary rates and genotype dynamics were also analysed, demonstrating changes in viral populations. Furthermore, we show that HMPV strains harbouring both recently discovered duplications co-circulate in Croatia and that these viruses belong to a subcluster A2c, which has completely replaced all other group A subclusters in 2017.

2. Materials and methods

2.1. Clinical samples

Clinical samples were nasopharyngeal secretions obtained from patients with ARI hospitalized in Croatia between March 2014 and April 2017. They were archived at the Croatian National Institute of Public Health within 24 h of submission. Presence of HRSV and HMPV in these samples has been detected by a direct immunofluorescence assay (DFA Light Diagnostics, EMD Millipore Corporation, Temecula, CA, USA) (Mlinarić-Galinović et al., 1987). Clinical samples positive for HRSV or HMPV in DFA were kept at −60 °C or below until molecular analysis. The study was approved by the Ethics Committee of the Croatian National Institute of Public Health, University Children's Hospital Zagreb, and University Hospital for Infectious Diseases (approval no. 01-692-1-2014, valid until 31st August 2018) and Ethics Committee of Medical School University of Zagreb (approval no. 380-59-10106-16-20/293, valid until 28th June 2018).

2.2. Reverse transcription, PCR and sequencing

Total RNA was extracted from 500 µL of clinical samples by the method reported by Chomczynski and Mackey (1998). Reverse transcription was performed at 42 °C for 60 min, in a reaction mix containing 10 µL of isolated RNA, 1× PCR buffer (GE Healthcare, UK), 0.1 mM of each dNTP, 20 U of RNase inhibitor (Thermo Fisher Scientific, USA), 1.25 mM $MgCl_2$, 2.5 mM of random hexanucleotide primers and 50 U of MuLV reverse transcriptase (Thermo Fisher Scientific, USA) in a final volume of 20 µL.

Nested PCR was performed for amplification of full length HRSV SH and HMPV SH and G genes as well as fragments used for genotyping, as reported in Ivancic-Jelecki et al. (2018), Jagušić et al. (2017) and Slovic et al. (2016). Full length HRSV G gene was amplified in two overlapping fragments using forward SH1 (5'-CACAGTKACTGACAAYAAAGGAGC-3′) and reverse G2 primer (5'-GAACACTTCAAARTGRWAATCAT-3′) for the first fragment, and forward G3 (5'-ATGATTWYCAYTTTGAAGTGTTC-3′) and reverse F164 (5'-GTTAT GACACTGGTATACCAACC-3′) for the second fragment, respectively. Final reaction mixtures contained 1× OneTaq PCR buffer (New England Biolabs, USA), 10 mM dNTP, 0.25 mM $MgCl_2$, 0.25 mM of each primer, and 1.25 U of OneTaq DNA polymerase (New England Biolabs, USA). The amplified products were separated on a 1.5% agarose gel, excised and purified by centrifugation through glass wool, as reported by Sun et al. (2012).

Sequencing reactions were set up with purified DNA, one of the specific primers used in the second PCR, and a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Sequencing and sequence analysis were performed on a 3130 Genetic Analyser (Thermo Fisher Scientific, USA).

2.3. Phylogenetic analysis

HRSV positive samples were genotyped by amplifying HVR2 of the G gene located at the protein's C-terminus. This region corresponds to nt 5274–5543 of strain A2 (acc. no. M11486) and 652–981 of BA4128/99B (acc. no. AY333364.1), prototype strains of group A and B, respectively. HMPV positive samples were genotyped by amplifying a 473-nt long region of the F gene which corresponds to nt 3680–4152 in genotype A reference strain CAN97-83 (acc. no. AY145296), and nt 3677–4149 in genotype B reference strain CAN98-75 (acc. no. AY145289).

Nucleotide sequences of HRSV and HMPV A and B strains of the genotypes previously described in the literature were obtained from the GenBank and used to construct alignments and phylogenetic trees. Alignments were performed using ClustalX 2.1 software (Larkin et al., 2007) and selection of the most suitable substitution model was determined with jModelTest 2.1.4 software (Darriba et al., 2012). Bayesian Markov Chain Monte Carlo (MCMC) inference was performed with BEAST v1.8.2 (Drummond et al., 2012). Convergence was assessed based on the effective sample size using Tracer v1.5 (http://beast.bio.ed.ac.uk/Tracer) after a 10% burn-in, and only values above 200 were accepted. Maximum clade credibility trees were generated with TreeAnnotator v1.8.2 and visualized with FigTree v1.4.2.

The sequences of HRSV strains obtained in this study were deposited in the GenBank under acc. nos. KX497066 – KX497131 and MH606020 – MH606143; and HMPV strains under acc. nos KX497048 – KX497065, MF104558 – MF104587, MH918029 – MH918060, MH713759 –

MH713780, and MK947186 – MK947209.

2.4. Phylodynamic analysis

Evolutionary rates of HRSV and HMPV complete SH and G genes were estimated using a Bayesian MCMC approach implemented in Beast v1.8.2. (Drummond et al., 2012). Sequences were trimmed using AliView software version 1.23 (Larsson, 2014). Rates and time of the most recent ancestor (tMRCA) were estimated using the relaxed (lognormal uncorrelated) molecular clock model. To estimate genetic diversity of a population over time, a Bayesian skyline plot analysis was performed. Convergence was assessed based on the effective sample size using Tracer v1.5 after a 10% burn-in, and only values above 200 were accepted. The uncertainties of the estimates were indicated by 95% highest posterior density (HPD) intervals. Complete datasets containing full-length SH and G gene sequences of HRSV and HMPV strains are available upon request.

2.5. Analysis of deduced amino acid sequences

Deduced amino acid sequences were generated using the standard genetic code implemented in MEGA software. NetOGlyc 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/) was used to predict potential O-glycosylation sites and NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) for N-glycosylation prediction.

2.6. Selective pressure analysis

Codon-based analysis of selective pressure was performed using the HyPhy package available on the Datamonkey server (http://www.datamonkey.org/) (Delport et al., 2010; Kosakovsky Pond et al., 2005; Kosakovsky Pond and Frost, 2005). Four different methods were used: single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), mixed effects model of evolution (MEME) and fast unconstrained Bayesian approximation (FUBAR) to avoid false-positive rates. Sites were considered positively or negatively selected if they met the cut-off criteria of a p-value of < 0.05 (for SLAC, FEL and MEME) and FUBAR posterior probability of > 0.90.

3. Results

3.1. Circulation patterns and phylogenetic analysis of HRSV and HMPV

From a total of 7586 patients diagnosed with ARI between March 2014 and April 2017, HMPV infection has been confirmed in 247 (3.2%) and HRSV infection in 1151 (15.1%) patients using DFA assay. Based on availability of biological material, further genotyping was performed on 185 HMPV-positive samples and 781 HRSV-positive samples; HMPV genome was identified in 156 (84.3%) and HRSV genome in 689 (88.2%) samples. The characteristics of patients from whom the samples were obtained in respect to diagnosis and age are given in Table S1. HRSV primarily caused severe illness in children under 7 months of age, while the largest number of HMPV infections was detected in children between 1 and 2 years old. Bronchiolitis was the most common diagnosis for HRSV patients and upper respiratory tract infection for HMPV patients (Table S1).

Characteristic seasonal distribution of largely overlapping HRSV and HMPV epidemics has been confirmed (Fig. 1) as well as the existence and co-circulation of groups A and B for both viruses (Figs. S1 and S2, Table 1). A similar overall number of HRSV infections were caused by group A (49.3%) and group B strains (50.7%); group A strains being predominant in 2015 and 2016 and group B in 2014 and 2017 (Table 1). During the analysed period, 104 (66.6%) of HMPV strains belonged to group A and 52 (33.3%) strains to group B; group A viruses were predominant in 2015 and 2017, while group B viruses took prevalence in 2014 and 2016 (Table 1). A gradual replacement of A2b HMPV strains with newly occurring A2c strains was observed, resulting in all infections in 2017 being caused by A2c.

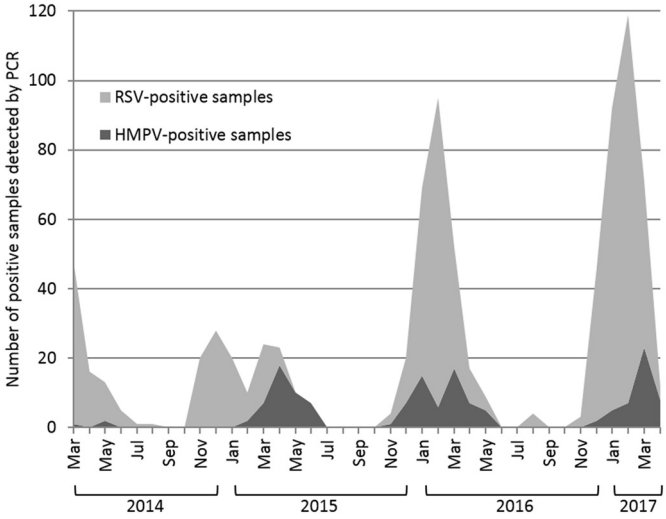

Fig. 1. Distribution of HRSV- and HMPV-positive samples detected during seasonal outbreaks in Croatia from March 2014 until April 2017.

Table 1
Distribution of HRSV and HMPV genotypes/subgroups.

Virus	Genotype/Subcluster	Year				Total
		2014	2015	2016	2017	
HRSV	NA1	0	5	31	0	36
	GA5	2	1	0	0	3
	ON1	55	36	145	65	301
	BA10	2	1	0	0	3
	BA9	70	23	67	186	346
	Total	129	66	243	251	689
HMPV	A2b	0	41	4	0	45
	A2c	1	7	18	33	59
	B1	1	3	14	6	24
	B2	1	8	15	4	28
	Total	3	59	51	43	156

3.2. Diversity of HRSV and HMPV strains

Diversity of HVR2 was calculated on the intra-genotype level for HRSV strains. Genotype BA9 showed the greatest diversity, comprising 96 different strains that shared 61.8% identity at the nucleotide level, followed by 90 unique ON1 strains that were identical at 63.5% positions. Even greater differences were observed at the amino acid level, the identities between strains dropped to 37.4% and 43.2% for BA9 and ON1, respectively. Strains belonging to NA1, GA5 and BA10 genotypes were nearly identical at the nucleotide level, with identities of 99% for all three genotypes.

In contrast, since a fragment of 473 nt of the F gene is used for HMPV genotyping, high degree of conservation was observed between strains, with identities above 90% for all clusters/subclusters. The most diverse strains belonged to A2c subcluster, where 30 unique strains shared overall identity of 91.5%, while A2b subcluster was highly conserved with identity of 96.1% between strains. In group B, identity of 94% was calculated within both clusters. Higher level of conservation was observed at the amino acid level with calculated identities of 97.5% for A2b, 95.5% for A2c, 100% for B1 and 97.5% for B2.

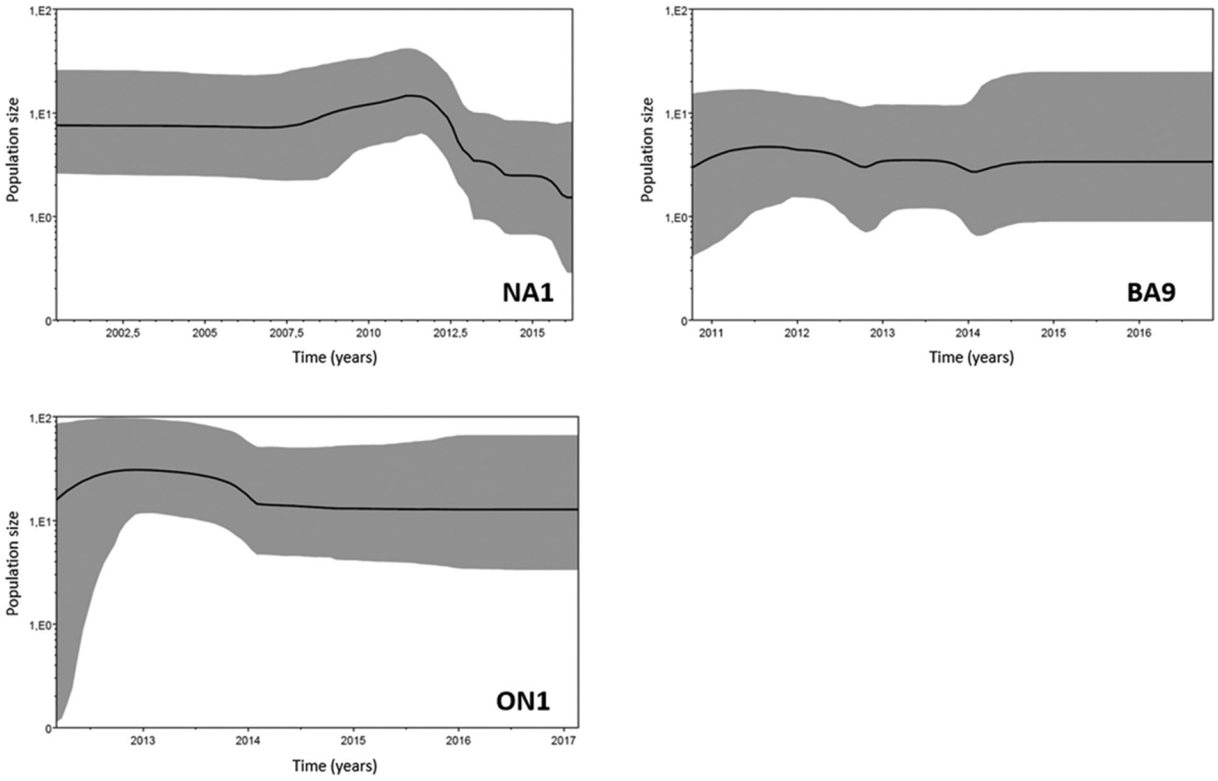

Fig. 2. Bayesian skyline plots showing demographic history of NA1, ON1 and BA9 genotypes. The TN93 model was implemented for NA1 (46 sequences) and ON1 (32 sequences), while HKY was used for BA9 (17 sequences) strains. Both models included gamma distributed rate variation among sites. Samples were run for 50, 45 or 30 million MCMC chains with sampling every 5000, 4500 and 3000 generations for NA1, ON1 and BA9 genotypes, respectively. The estimated change in effective population size over time is indicated. The thick lines represent the mean estimate, 95% highest-posterior-density intervals are represented by the grey area.

3.3. SH and G gene-based evolutionary dynamics of HRSV and HMPV strains

Bayesian MCMC analysis was used to estimate nucleotide substitution rates based on sequences of complete SH and G genes of HRSV and HMPV for which the exact date of collection was known. In order to obtain more conclusive results, the analysis also included samples from years 2011 to 2014. Complete datasets containing full-length SH and G gene sequences of HRSV and HMPV strains are available upon request.

For HRSV, analysis included strains belonging to NA1 (46 sequences), ON1 (32 sequences), and BA9 (17 sequences) genotypes whereas underrepresented GA5 and BA9 strains were left out due to their low number. As previously described in Slovic et al. (2016), Bayesian skyline plot for genotype NA1 strains showed that population remained constant until 2011 and started to rapidly decease in the following years (Fig. 2). Conversely, a sharp increase in population size during 2011 and 2012 was demonstrated for genotype ON1 strains followed by a slight decrease in 2013 and a constant size from this point forward (Fig. 2). Except for minor fluctuations at the end of 2012 and the beginning of 2014, a population size of BA9 genotype strains did not significantly change (Fig. 2). Estimated substitution rates, calculated as nucleotide substitutions per site and year, for both group A genotypes (NA1 and ON1) were approximately 2.5×10^{-3}, whereas slightly higher rate (3.53×10^{-3}) was observed for BA9 genotype strains (Table 2).

Phylodynamic analysis of HMPV included group A (26 sequences) and group B (20 sequences) strains; analysis was not possible on the subcluster level due to low number of samples. As demonstrated by Bayesian skyline plot, a population size of group A HMPV strains remained constant until a second half of 2008 when a sharp increase in population size was observed. From 2010 onwards, group A population size remained constant (Fig. 3). At the same time, a decrease in population size of group B was noticed followed by an increase in 2011 to 2013, and again reduction in population size in 2014 (Fig. 3). Similarly to HRSV strains, estimated substitution rate of HMPV group A strains was 2.51×10^{-3} and slightly higher rate (3.61×10^{-3}) was calculated for group B strains (Table 2).

3.4. Duplication events

Duplication in HRSV ON1 strains was extensively analysed in our previous work (Ivancic-Jelecki et al., 2015; Slovic et al., 2016). Therefore in this paper, emphasis was given to novel HMPV duplications.

Out of 46 HMPV samples collected from 2011 to 2016 which were included in our phylodynamic analysis, two HMPV strains belonging to A2c subcluster (strain HR900-15, acc.no. MH918037 and strain HR1119-16, acc.no. MH918060) that originated from April 2015 and April 2016 were found to contain a large, 180-nt long, duplication in the extracellular part of the G gene (nucleotide positions 371–550). This, as well as one shorter duplication (111-nt long, positions 421–531), were recently found in the G genes of HMPV group A strains circulating in Japan (Saikusa et al., 2017a; Saikusa et al., 2017b) and Spain (Piñana et al., 2017), annotated as subcluster A2b and A2b2 strains, respectively. Therefore, extracellular region of strains genotyped as subcluster A2b in this study (based on F gene fragment) was also checked for duplication: 45 of a total of 53 strains originating between 2011 and 2016 were sequenced and none of the strains contained either duplication. We therefore extended our HMPV G gene analysis on group A strains isolated in 2017 (all of them were A2c strains, A2b strains were not detected) which were available for analysis in sufficient amount. A total of 18 (from 33 A2c strains) complete G gene sequences were obtained (acc. Nos. MK947192-MK947209); two (11.1%) of them did not contain any duplication, 11 (61.1%)

Table 2
Nucleotide substitution rates calculated as substitutions per site and year and tMRCA estimates based on sequences of complete SH and G genes of HRSV and HMPV.

Virus	Group	Subcluster/Genotype	Nucleotide substitution rates		tMRCA	
			Mean	95% HPD	Mean	95% HPD
HRSV	A	NA1	2.55×10^{-3}	$1.59–3.48 \times 10^{-3}$	1990	1978–2000
		ON1	2.58×10^{-3}	$1.64–3.61 \times 10^{-3}$	2011	2009–2012
	B	BA9	3.53×10^{-3}	$2.39–4.85 \times 10^{-3}$	2009	2007–2010
HMPV	A	A2a and A2b	2.51×10^{-3}	$1.75–3.38 \times 10^{-3}$	1994	1986–2001
	B	B1 and B2	3.61×10^{-3}	$2.26–5.00 \times 10^{-3}$	1973	1954–1990

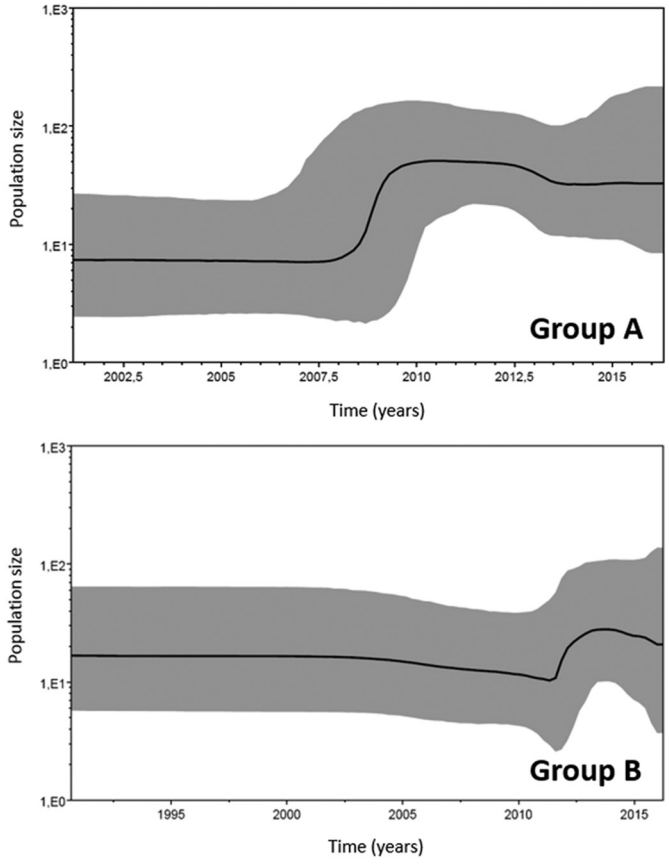

Fig. 3. Bayesian skyline plots showing demographic history of HMPV group A and B. The TN93 model with gamma distributed rate variation among sites was implemented. Samples were run for at least 50 million MCMC chains, with sampling every 5000 generations. The estimated change in effective population size over time is indicated. The thick lines represent the mean estimate, 95% highest-posterior-density intervals are represented by the grey area.

contained 180-nt long duplication and five (27.7%) contained 111-nt long duplication. Phylogenetic analysis based on available sequences from the GenBank showed that Croatian strains with duplication clustered with Japanese and Spanish strains whether the F gene fragment or complete G gene was used for tree reconstruction (not shown). Thus, we genotyped the strains as A2c, according to nomenclature by Nidaira et al. (2012).

Both duplications were in-frame and they extended the G protein by 37 or 60 amino acids, respectively (Figs. 4 and 5). Only three strains with 60-amino acids long duplication had the same sequence in both segments (HR129-17, MK947192; HR1219-17, MK947203; and HR1392-17, MK947209). Maximum number of differences in duplicated segments was five (found in strain HR906-17; MK947197). Analysis of six strains with 37-amino acid duplication revealed that no strain had the same two copies (Figs. 4 and 5). Even in the first detected strain with 111-nt duplication (HR761-17, acc. no. MK947195), two amino acid differences were present (Fig. 4).

Since Croatian strains bearing duplications have appeared around the same time as Japanese and Spanish strains, we compared their sequences. Despite being geographically distant, Croatian strains with 180-nt duplication were closer to Japanese strains; there was only one amino acid difference between Croatian strain HR900-15 and Japanese strain P7916 (acc. no. LC192250.1). The observed difference (S174A, all changes are shown relative to Croatian strain) is located in the 1st copy of the duplicated segment (Fig. 4). In regard to Spanish strains, the closest match was found between HR900-15 and strain NSVH2016-15-56796 (acc.no. AQW35134.1). These strains differ by a total of three amino acids which were all found within copies of duplicated segment (S174A, A242T and T243I).

A second duplication of 111-nt has so far been described only among viruses circulating in Japan, where three strains were found to contain this shorter duplication. All strains were isolated in May 2017, while first strain that possesses such duplication among Croatian viruses originates from February of the same year. Comparison of their sequences revealed that the smallest number of differences is observed between strain HR1495-17 (acc.no. MK947210) and Japanese strains P8943 (acc.no. BAX90169.1) and P8945 (acc.no. BAX90170.1). These strains differ in 12 positions, four of which are within duplicated segment (F162L, R187K, S195P and L199F).

Potential glycosylation sites were analysed for HMPV strains containing the 37-amino acids long and 60-amino acids long duplications. In contrast to strains without duplication, these strains possess 16 (37-amino acids duplication) and 25–26 (60-amino acids duplication) novel potential O-glycosylated sites. New potential N-glycosylation sites were not identified within duplications.

3.5. Analysis of deduced SH- and G-protein amino acid sequences of HRSV and HMPV strains

Codon-based selective pressure analysis of deduced SH and G proteins detected positive selection only in HRSV group A and G proteins of both HMPV groups (Table S2). Comparison of dN/dS values within HRSV strains showed G proteins of NA1 strains are more diverse than BA9 and ON1 strains. HMPV strains of both major groups had similar dN/dS value, indicating their similar overall diversity (Table S2). In contrast, SH proteins of both viruses were conserved, as shown by their low overall dN/dS value (Table S2).

Glycosylation pattern of both viruses was examined for major HRSV genotypes and HMPV groups, respectively, and is given in Table S3. Analysis of N-glycosylation pattern of HRSV G proteins showed genotype NA1 had the greatest number of potential acceptor sites. In contrast, only three sites were predicted to be glycosylated in ON1 strains, of which no site was within 24 amino acids duplicated region. HRSV SH proteins of all three genotypes had a single potential N-glycosylated position. The number of N-linked glycosylation sites present in the HMPV G protein varied from one to four and retained group-specific pattern, while glycosylation pattern of SH protein was completely conserved in group A (Table S3). Due to large number of possible acceptors of O-linked glycans in the G proteins of both viruses, their glycosylation profile was not examined.

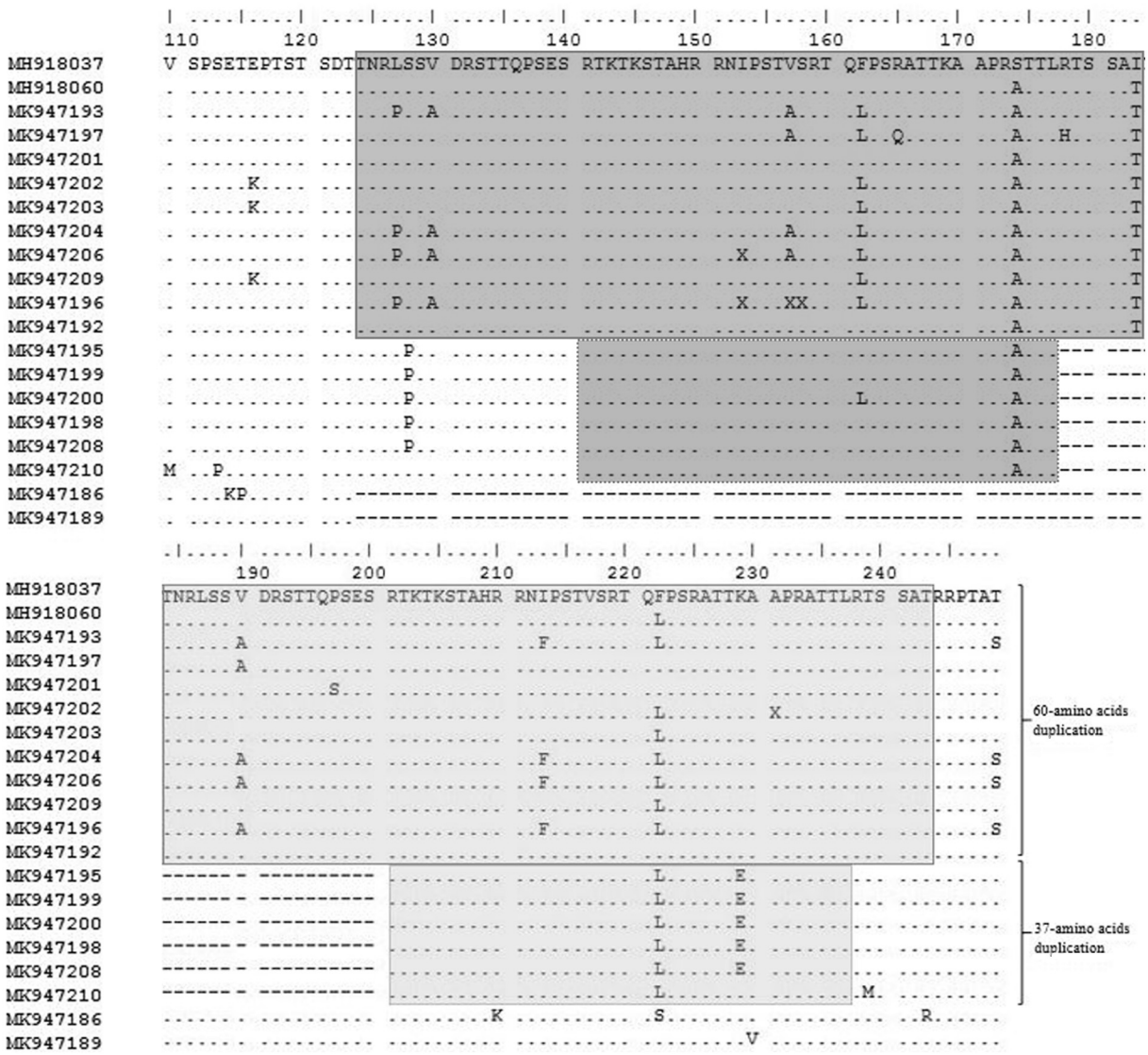

Fig. 4. Alignment of deduced amino sequences of HMPV group A G proteins shown relative to the sequence of strain MH918037. Region spanning positions 110–250 is shown. Duplicated regions are indicated with shaded rectangles: darker grey indicates 1st copy and lighter shade 2nd copy of the duplicated region. Identical residues are indicated by dots, dashes designate gaps and asterisks indicate stop codons.

Fig. 5. Frequency pictograms of a 60-amino acids long (a) and 37-amino acids long (b) duplicated region in the G gene of HMPV A2c strains. The upper panel in each block shows a sequence of the first copy and the lower panel shows a sequence of the second copy of the duplicated segment. The asterisks mark the positions that differ between the 2 copies of the duplicated segment.

4. Discussion

The present study describes HRSV and HMPV strains circulating in Croatia during a 4-year period (2014–2017), providing insight into genetic variability and evolution of these viruses. Multiple different strains were shown to co-circulate and comparisons to previous epidemiological data were made. In addition to presenting genetic versatility of pneumoviruses in this geographic region, evolutionary dynamics based on complete SH and G genes of selected strains was also analysed. In this study novel HMPV viruses containing large duplications in the G gene were detected, which have so far been detected only in Japan (Saikusa et al., 2017a, 2017b) and Spain (Piñana et al., 2017).

Seasonal peaks of HRSV and HMPV are inversely proportional, that is in years when more HRSV cases are detected during winter months (*e.g.* 2014/2015 season, Fig. 1), a smaller spring epidemics of HMPV follows. This pattern has already been described in literature (Ljubin-Sternak et al., 2014). Since these viruses can induce cross-reactive antibodies (Wen et al., 2017; Zhang et al., 2015), shared antigenicity and immunologic cross-protection have been proposed as possible reasons for this phenomenon (Zhang et al., 2015), similarly to human parainfluenza viruses types 1 and 3 (Fry et al., 2006).

Phylogenetic analysis of HRSV revealed that Croatian HRSV A strains were classified within NA1, ON1 and GA5 genotypes, while BA9 and BA10 genotypes were detected among HRSV B strains. In contrast to our previous study in which NA1 was the dominant group A genotype (Slovic et al., 2016), genotype ON1 gained strong dominance in period 2014–2017. Furthermore, of 36 NA1 strains the majority possessed identical HVR2 sequence (Fig. S1) which could indicate that these strains come from the same source, since it is known that multiple introductions would result in higher diversity of viral groups (Shiino, 2012). As expected, diversification of ON1 strains happened with the expansion of the ON1 population size. The greatest diversity was observed within BA9 strains, although no positively selected sites were present in G proteins of BA9 strains, suggesting that the majority of observed substitutions are neutral.

Similarly, circulation of multiple lineages of HMPV was observed within both major HMPV groups. Two subclusters were detected in group A, A2b and A2c, with predomination of subcluster A2c strains, while group B subgroups B1 and B2 caused similar number of infections. Contrary to HRSV, results from this and our previous studies (Jagušić et al., 2017; Slovic et al., 2016) suggest a change in predominant HMPV group happens in subsequent years, while HRSV maintains a biennial pattern for group A, followed by a year in which group B predominates. In contrast to our previous study (Jagušić et al., 2017), subcluster A2a strains were not detected. Since this subcluster has not been detected in Croatia since 2012, it may be that newly emerging strains have replaced this lineage, an outcome previously shown for other HMPV lineages (Aberle et al., 2010; Huck et al., 2006). It seems that newly occurring A2c strains are gradually replacing A2b HMPV strains resulting in all infection being caused by A2c genotype in 2017. Of note, only A2c genotype has been detected in our ongoing surveillance study during 2018 (unpublished data). Furthermore, A2c strains showed the greatest diversity and duplication events have been detected only among A2c strains (see below).

The evolution of HRSV was analysed for three prevalent genotypes based on the complete SH and G genes collected during 2011–2016. Similar evolutionary rate of approximately 2.5×10^{-3} substitution/site/year was obtained for both group A genotypes, in contrast to our previous study in which ON1 genotype had much higher rate (Slovic et al., 2016). The difference arose as a result of previous calculation using only highly diverse HVR2 fragment, as it is know that HRSV G protein has significantly higher rate than the rest of the genome (Schobel et al., 2016; Tan et al., 2013). The difference can also be attributable to the fact that ON1 genotype was newly introduced genotype at the time of the previous study so higher local rates can be expected due to local host population immunological differences (Duvvuri et al., 2015). Substantially higher evolutionary rate was estimated for the BA9 genotype (3.5×10^{-3} substitution/site/year), which is consistent with the faster evolutionary rates reported for group B viruses (Schobel et al., 2016; Tan et al., 2013). Genotype shift in the circulating HRSV group A population was evident from Bayesian skyline plots: a dramatic decrease in genetic heterogeneity of NA1 strains starting from 2011 was accompanied with an increasing population size trend observed for ON1 strains. Similar results have been reported by Zhang et al. (2018) who showed a decline of group A population in 2012, followed by its rapid increase in subsequent year when ON1 strains appeared. The authors also showed that group B population size was more stable over the analysed period, due to constant circulation of BA9 strains (Zhang et al., 2018). We found the same for Croatian group B strains, demonstrating that cyclic pattern of HRSV group predominance, shown by Mlinaric-Galinovic et al. (2012) and Trento et al. (2010) is not affected by introduction of a novel genotype.

It has previously been shown that most HMPV genes regardless of a genetic lineage show relatively low genetic diversity (de Graaf et al., 2008; Kim et al., 2016). In our study, evolutionary dynamics of HMPV was inferred from complete sequences of SH and G genes, which generates a fragment of 1463 nt for group A, and 1481 nt long for group B. Previous evolutionary studies were based on a much shorter regions of these genes, *e.g.* de Graaf et al. (2008) inferred a rate of 3.5×10^{-3} substitutions/site/year based on 442 nt of G gene, and in a study performed by Kim et al. (2016) calculated rate for SH gene (531 nt) was 1.5×10^{-3}, while inferred rate for 444 nt of G gene was 2.13×10^{-3} substitutions/site/year. Our results are not in full agreement but fall within 95% HPD of rates inferred in both studies. de Graaf et al. (2008) showed that tMRCA based on G gene of two main lineages, group A and B, is strikingly similar, whereas in our study group A lineage was shown to be of a more recent origin than group B lineages. Changes in effective population size were observed in both groups, although these were transient. A decrease in genetic diversity was noticed at the end of 2014 for group B and the low population level continued onwards. Further studies are needed to clarify if this trend continues, possibly accompanied with an increase in group A effective population size, due to newly introduced A2c strains.

Sixteen HMPV strains were found to contain large duplications in the extracellular domain of the G protein: 11 strains had 180-nt and five strains had 111-nt duplication, respectively. Up until now, most of the observed length variation of the HMPV G protein was due to substitutions and frameshift mutations (Agrawal et al., 2011; van den Hoogen et al., 2004) possibly occurring as a result of immunogenic pressure in the same manner as was postulated for the HRSV G protein (Cane and Pringle, 1995). Since these large duplications extend G protein by 37 or 60 amino acids, it has been proposed that additional amino acids help in immune evasion through enhanced shielding of the F protein (Saikusa et al., 2017a). Additionally, extra glycans present in the duplicated region may also be advantageous for these strains, either by enhancing binding as was proposed for HRSV (Hotard et al., 2015) or through evasion of immune response (Sommerstein et al., 2015). Since HRSV genotypes containing significantly smaller duplications have rapidly become dominant, it will be interesting to see whether this happens in the near future with HMPV as well. Recent findings suggest a beneficial role of 111-nt duplication, as strains with this duplication have already become predominant in Yokohama city, Japan in 2018 (Saikusa et al., 2019).

In conclusion, co-circulation of multiple lineages was observed for both viruses. Within HRSV group A, ON1 strains gained strong predominance during the 4-year period, while previously dominant genotype NA1 was detected only sporadically. Similarly, newly occurring HMPV genotype A2c gained predominance over genotype A2b during this period, resulting in all infection in 2017 being caused by A2c. Evolutionary dynamics showed that inferred substitution rates of HRSV and HMPV are similar and substantially higher rates were observed for group B of both viruses. This study established presence of recently

described HMPV strains containing large duplications in the G gene in Croatia, showing these strains are already spreading globally.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.104039.

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