Trends and Intensity of Rhinovirus Invasions in Kilifi, Coastal Kenya Over a Twelve-Year Period, 2007-2018

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ABSTRACT

Background: Rhinoviruses (RVs) are ubiquitous pathogens and the principal etiological agents of common cold. Despite the high frequency of RV infections, data describing their long-term epidemiological patterns in a defined population remain limited.

Methods: Here, we analysed 1,070 VP4/VP2 genomic region sequences sampled at Kilifi County Hospital on the Kenya Coast. The samples were collected between 2007 and 2018 from hospitalised paediatric patients (< 60 months) with acute respiratory illness.

Results: Of 7,231 children enrolled, RV was detected in 1,497 (20.7%) and VP4/VP2 sequences were recovered from 1,070 samples (71.5%). A total of 144 different RV types were identified (67 *Rhinovirus A*, 18 *Rhinovirus B* and 59 *Rhinovirus C*) and at any month, several types co-circulated with alternating predominance. Within types multiple genetically divergent variants were observed. Ongoing RV infections through time appeared to be a combination of (i) persistent types (observed up to seven consecutive months), (ii) reintroduced genetically distinct variants and (iii) new invasions (average of eight new types, annually).

Conclusion: Sustained RV presence in the Kilifi community is mainly due to frequent invasion by new types and variants rather than continuous transmission of locally established types/variants.

Key words: Rhinovirus, coastal Kenya, long-term surveillance, persistence, invasion.

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Introduction

Rhinovirus (RVs) are a highly prevalent group of viruses and are the principal cause of common cold syndrome in humans globally [1,2]. RV infections result in a wide range of clinical outcomes spanning from asymptomatic and mild illness in the upper airways to severe illness in the lower airways [3,4]. The infections occur in all ages, with severe presentation more likely in children under the age of 5 years [5,6], the elderly [7] and immunocompromised [8]. Despite the clinical significance of RV infections, there is little information on the long-term trends and diversity of circulating RV types.

RV belongs to the genus *Enterovirus* of the family *Picornaviridae.* The viral single-stranded positive sense RNA genome consists of ~7200 nucleotides and encodes four structural proteins (VP4, VP2, VP3 and VP1) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) [2]. The three surface-exposed capsid proteins (VP1, VP2 and VP3) carry the antigenically critical sites [9–11]. The high genetic variability in the VP4/VP2 and VP1 genomic regions of rhinoviruses have been instrumental in molecular typing [12,13] and molecular epidemiological investigations of rhinovirus infections [14–16]. Currently, a total of 169 RV types have been described, and classified into three species i.e., *Rhinovirus A, Rhinovirus B* and *Rhinovirus C* (https://www.picornaviridae.com/sg3_ensavirinae/enterovirus/enterovirus.htm).

RV infections occur all year-round in most geographical locations, although peaking in the early autumn and late spring in many temperate countries, and in the rainy season in tropical countries [2,17]. Unclear seasonality and year-round transmission of rhinoviruses have been attributed to lack of inter-type cross-protective immunity [18,19], coupled with the high genetic diversity within the three species, each with the ability to spread independently in a population [16,20,21].

A recent study in Kilifi county located in coastal Kenya, that span over 12-month period [21] (December 2015 - November 2016), found that multiple RV types co-circulate over

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varied time periods ranging from 1 to 9 months and in most cases, each displaying a typical epidemic curve at the local population level; transmission presumably constrained by the decline in susceptibles to that type within the locality. Type-specific (homologous) immunity has been reported to wane approximately after a one-year period [22], and individuals who were previously immune to a particular type gradually become susceptible to the type again [22,23]. Previous studies found that introduction of new RV types or sequential invasion by different genetic variants could be due to declining levels of population immunity as well as viral evolution [24,25].

These assertions of perpetually changing RV types during year-round RV transmission have not been fully investigated in a longitudinal manner [16]. In this study, we analyzed VP4/VP2 sequences of samples collected from hospitalized children with acute respiratory illness (ARI) between 2007 and 2018 on the Kenyan Coast to evaluate the long-term incidence of the different RV types, their temporal patterns and intensity of new invasions in a local population.

Methods

Study area and population

The study was conducted at the Kilifi County Hospital (KCH) as part of long-term surveillance initially aimed at understanding the epidemiology and disease burden of respiratory syncytial virus (RSV)-associated pneumonia cases [26] and expanded to a range of respiratory viruses from 2007 [27–32]. KCH, located on the coast of Kenya, is a referral hospital serving the wider Kilifi County which has a population of 1,453,787 and covers an area of ~12,254 square km. Details of study design, participant recruitment, and sampling procedures have been described elsewhere [26,32]. Briefly, upon presentation to the paediatric ward, a detailed medical review was undertaken by the clinician and the decision to admit was made. For this study, children (<60 months of age) admitted to the paediatric ward

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between January 2007 and December 2018 were eligible if they presented with symptoms of syndromic severe or very severe pneumonia. Clinical definitions include: a history of cough or difficulty in breathing for less than 30 days, which if accompanied by lower chest wall indrawing was defined as severe pneumonia; or if accompanied by any one of prostration, coma or hypoxemia was defined as very severe pneumonia; prostration including the inability to feed or drink, and hypoxemia defined by oxygen saturation (pO_2) <90% [26]. Following a written informed consent from the parent or guardian, a nasopharyngeal flocked swab, nasal wash or combination of nasopharyngeal swab and oropharyngeal swab was collected from each child and transferred into viral transport medium for laboratory screening. Ethical approval for the study protocol was obtained from the Scientific and Ethics Review Unit (SERU # 3443) ethics committee, Kenya Medical Research Institute, Nairobi, Kenya.

RV screening and sequencing

Viral RNA was extracted from each sample using QIAamp Viral RNA kit (Qiagen Inc., Valencia, California, USA) and screened for respiratory viruses using a multiplex real-time reverse-transcription PCR (rt-RT-PCR) (Applied Biosystems, United Kingdom) as described elsewhere [33,34]. A sample was considered RV positive if rt-RT-PCR cycle threshold (Ct) was <35.0 [32]. A section of VP4/VP2 viral genomic region (~420 nucleotides long) of positive samples was amplified and sequenced as previously described [21]. Consensus sequences were assembled using the Sequencher software version 5.4.6 (Gene Codes Corporation, Ann Arbor, USA).

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Sequence data, RV species and type assignment

VP4/VP2 sequencing, and typing was attempted for all the RV positive samples collected in 2014, 2016 to 2018. For the years 2010 to 2013 and 2015, 100 RV positive samples were randomly selected for sequencing proportional to the monthly distribution of positive samples (Supplementary Table 1). Previously published VP4/VP2 sequences from Kilifi (January 2007– December 2009) were retrieved from GenBank (n=271, sequence accession numbers: KY006195 - KY006465) and combined with the 799 newly generated VP4/VP2 sequences (January 2010 – December 2018, GenBank sequence accession numbers; MW622248 - MW623046).

Definition of terms

We used the term 'type' to refer to RV sequences classified by either cross-neutralization or genetic comparisons as distinct as described previously [13]. Based on this approach, sequences were assigned into the same RV type based on >90% nucleotide similarity to rhinovirus prototype sequences (also referred to as reference sequences, http://www.picornaviridae.com/sequences/sequences.htm) and phylogenetic clustering with bootstrap support value >70% [13]. Distributions of pairwise genetic distances were assessed for evaluation of intertype and intra-type divergence [13]. Intra-type 'variant' was defined on the basis of a divergence threshold value determined as the least frequent value between the first and second modes in a pairwise nucleotide difference distribution plot. Here we are implicitly assuming that sequences with pairwise nucleotide difference falling into the distribution with the low (first) mode are members of the same phylogenetic clade, whereas those with pairwise nucleotide difference within the second distribution with higher mode are members of different phylogenetic clades. A group of viruses within the first, lower distribution were classified as belonging to the same RV type variant.

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The definitions used to describe the temporal occurrence of RV types are summarised as follows:

a) Persistent: Continued detection, in consecutive or non-consecutive years, of a group of viruses belonging to the same variant of a RV type.
b) Recurrent: Detection of a virus or group of viruses not observed in the preceding years (>1 year) that belong to a different variant of a previously observed RV type.
c) Invasion: Detection of a new RV type not previously locally documented.

Phylogenetic analyses

Multiple sequence alignments (MSA) were generated using MAFFT v7.220 [35] and maximum likelihood phylogenetic trees estimated using IQ-TREE v1.6.12 [36]. Branch support was assessed by 1000 bootstrap iterations. Temporal signal in the data was examined using TempEst v1.5.3 [37]. To infer time-scaled phylogenies, Bayesian phylogenetic analyses were undertaken in BEAST v.1.10.4 assuming an uncorrelated lognormal relaxed molecular model [38]. The MCMC convergence was assessed in Tracerv1.5 and maximum clade credibility (MCC) trees summarized using TreeAnnotator v1.10.4 with a 10% burn-in. MCC trees were visualized using FigTree v1.4.4.

Results

RV prevalence in Kilifi, 2007 to 2018

Between January 2007 and December 2018, a total of 7,231 NPS samples were collected from children (<60 months of age) admitted with severe or very severe pneumonia in KCH (Supplementary Table 1). RV was detected in 20.7% (1,497/7,231), with the proportion positive across the years ranging from 15.6% to 38.3 % (Supplementary Table 1). The monthly frequency of detection of RV in the study population is shown in Figure 1. RV

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infections were observed to occur year-round frequently peaking between the month of May and September each year (Figure 1).

RV species and type assignment

A total of 1,070 (71.5%) VP4/VP2 sequences (~420 nucleotides, some previously reported [32]) were available for this analysis. Of these, 520 (48.6%) sequences were classified as *Rhinovirus A* comprising 67 distinct types; 52 (4.7%) sequences were *Rhinovirus B* comprising 18 types and 498 (46.5%) were *Rhinovirus C* comprising 59 types. *Rhinovirus A* and *Rhinovirus C* were more frequently detected while *Rhinovirus B* infections were low in number and sporadic (Figure 2(A)). The most commonly detected types were RV-A49 (n = 39), C2 (n = 29), C38 (n = 26), C11 (n = 26), A101 (n=24), A12 (n=23), C6 (n = 22), C21 (n = 21), C3 (n=20) and A78 (n = 19) (Table 1). Twenty-four sequences could not be assigned to known RV types based on the criterion proposed by McIntyre [13] due to these sequences having *p*-distance of >10.5% with respect to their closest reference sequences (Supplementary Table 2). Other enteroviruses were also detected on sequencing the rt-RT-PCR rhinovirus detections: enterovirus D68 (EV-D68) (n=5), coxsackievirus-B3 (CVB3) (n=1), coxsackievirus-B2 (CVB2) (n=1) and echovirus19 (E19) (n=1).

Temporal trends of RV types in Kilifi

We detected on average, 39 RV types annually (range, 35 - 47), a mean of eight (range, 1- 29) of which were new RV types identified for the first time in the population each year from 2008 as others previously detected types disappeared (Figure 2(B)). The cumulative number of new RV types detected annually increased rapidly since the beginning of the surveillance period and then saturated after approximately nine years (Figure 2(B)). RV types commonly co-circulated and with varying frequency in the 12-year period (Figure 2(C), Supplementary File 1). Several types were present at high prevalence while others occurred once or sporadically. Some types circulated consecutively for months: for example,

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RV-A56 was detected in 7 consecutive months (May to November 2007), RV-C11 was present for 6 consecutive months (February to July 2016), and RV-C38, A40 and C2 types circulated consecutively for 5 months (November 2009 to March 2010, April to August 2016 and May to September 2010, respectively) (Supplementary File 1).

Additionally, several types recurred after considerable periods of absence. For example, RV-A12 first seen in February 2007 was not detected again until February 2009, 23 months later, while C38 viruses were detected 4 years apart between 2012 and 2016 (Figure 3, Supplementary File 1). Temporally, several RV types exhibited synchronized co-circulation and recurrence, for example (i) RV-C1, C11, C2, C38, C22 and C21; (ii) RV-A75, A89, A12, A28, A96, A106, A80 and A10; (iii) RV-A90, A55, A61, A45, A54 and A60; (iv) RV-C14, C41, C45, C10, C16, C25, C32 and C47.

Genetic diversity of RV types in Kilifi

The nucleotide (nt) sequence identity among *Rhinovirus A, Rhinovirus B* and *Rhinovirus C* viruses was determined as 57.3–100%, 66.0–100%, and 45.1–100%, respectively, and 59.8–100%, 79.9–100% and 53.3–100% at the amino acid (aa) level, respectively. Intra-type nucleotide variation was observed in the VP4/VP2 region of viruses sampled over the 12 years study period (Figure 4(A), Supplementary Figure 1). Nonetheless, the substitutions were mostly synonymous i.e., not amino acid changing. The distribution of pairwise nucleotide distances showed multi-modal peaks suggesting circulation of distinct variants within individual RV types (Figure 4(B), Supplementary Figure 2). These observations were congruent with multiple within-type phylogenetic clusters.

Several RV types were characterised by genetically distinct temporal clusters, for example, RV-A49, C38 and A101 (Figure 5). RV-A49 was detected as eleven distinct variants circulating at different periods, three of which occurred as singletons (single sequences) suggesting under sampled genetic diversity (Figure 5, Table 2). Multiple genetic

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variants of type RV-C6 co-circulated during 2010/ 2011 (Supplementary Figure 3, Table 2), which likely indicates separate virus introductions into the Kilifi population. Several RV types had variants that contained sequences from multiple years indicating variant persistence over an extended period or repeated reintroductions, e.g., RV-A101 variant 5 (v5) comprised of viruses observed from 2010 to 2013 (Figure 5, Table 2).

Discussion

We describe the long-term pattern of co-circulation, persistence and invasion of rhinovirus types in hospitalized children (less than 60 months old) with pneumonia in Kilifi coastal Kenya over a 12-year period (2007 to 2018). Consistent with other studies, RV was ubiquitous and multiple types co-circulated even within a single month [16,20,21]. Among the RV cases detected, *Rhinovirus B* was least frequently detected. It is not clear why *Rhinovirus B* is less diverse and each type within it was on average less frequent. The observed annual proportions of RV species in Kilifi are consistent with recent similar epidemiological studies in Brazil, Nigeria and Cameroon [39–41]. Although children under five years of age are not a comprehensive representative of the community, this demographic gives insight into the pattern of RV transmission since RV burden is highest in children under 5 years [14]. Rhinovirus detection rates decrease with increasing age as adults have had multiple and widespread exposures to rhinovirus types [14]. Other social groups are vital in RV transmission [30,42,43] and it would be useful to evaluate rhinovirus transmission patterns and prevalence within these groups.

The majority (99%) of our sequences were within the proposed divergence thresholds for RV typing and classification using VP4/VP2 region (10.5% for *Rhinovirus A*, 9.5% for *Rhinovirus B*, and 10.5% for *Rhinovirus C*) [13]. This exemplifies significant sequence conservation in the VP4/VP2 region within a type allowing robust genotypic assignment. However, 24 sequences did not fit the classification system for VP4/VP2 region and require

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whole genome sequencing to check for variation in the VP1 region and determine if they correspond to new types [13]. Detection of other enteroviruses (EVs) reflects PCR cross-reactivity due to nucleotide conservation at the 5'-untranslated target region [44,45]. EV-D68, CVB3, CVB2 and E19 have been associated with respiratory disease or detected in respiratory samples [46,47].

The frequent invasions of new types could be explained by lack of pre-existing immune memory or weak heterotypic immunity [48]. The number of new types decreased over time, levelling off in 2016, perhaps indicating the period a population takes to experience the maximum number of RV types. Recurrence of RV types could be promoted by antigenic variation on the other surface proteins (VP2, VP1 and VP3) allowing infection where prior exposure confers incomplete or short-lived immunity to future genetic variants. Recurrence, particularly where the recurring strains were genetically identical to older strains, may also be observed in a population not previously exposed to a RV type. Some RV types occurred sporadically and could be associated with mild disease or asymptomatic infections or have reduced transmission rates probably suppressed by pre-existing immunity [49].

For some RV types, the sequenced VP4/VP2 region remained conserved after periods of quiescence, which probably ensures strain survival by maintaining low-level genetic variation. In a linear strain space, strains interact via cross-immunity to nearby strains with shared epitopes and this interaction tails off with genetic distance [50]. Yet, the VP4/VP2 region might not be primarily antigenic [51], and genetic changes could have occurred at immunogenic sites located in other capsid proteins (VP1 or VP3). Genome-wide sequence data would therefore be useful to confirm strain conservation and maintenance. The evident intra-type genetic diversity with differential temporal distribution could suggest sequential virus introductions or diversification of locally circulating variants [52].

This study had two limitations. First, in some years (2010 to 2013 and 2015) we only sequenced a proportion of the positive cases (Supplementary Table 1), which might

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underestimate the circulating rhinovirus diversity. Samples selected were prioritised based on viral load and monthly distribution. Second, we only sequenced the VP4/VP2 coding region, but more reliable phylogenetic relationships would be defined from full-length genome analysis [53].

In conclusion, this study describes the nature of RV infections in hospitalized children less than 60 months old and enhances our understanding on rhinovirus transmission dynamics in a community. Rhinovirus dynamics in Kilifi during 2007-2018 were characterised by repeated invasions by heterogeneous types rather than long-term continuity of the same RV types and continuous diversification of circulating variants. Improved understanding on the RV types circulating in a community may support better guidance of future therapeutic interventions in clinical practice. The high diversity and rates of invasion of RV as observed in this study, even within a short duration (week or month), underpins the application of molecular typing for surveillance and understanding virus epidemiological dynamics.

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Author Contributions

J.M.M, E.K., C.A., D.J.N., designed the study, J.M.M., C.L., R.C., M.M., C.O. performed rhinovirus screening and sequencing, J.M.M., E.K., M.M.L, N.M., curated and analysed data and J.M.M., E.K., C.A., D.J.N., wrote the manuscript. All authors have read and approved the final manuscript draft.

Patient Consent Statement

Patients written informed consent was sought from the parent or guardian. Ethical approval for the study protocol was obtained from the Scientific and Ethics Review Unit (SERU # 3443) ethics committee, Kenya Medical Research Institute, Nairobi, Kenya.

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Potential Conflicts of Interest

The authors declare that they have no competing interest.

Availability of data and materials

All data generated and analysis script for this manuscript are available from the Virus Epidemiology and Control (VEC), KEMRI-Wellcome Trust Research Programme, data server under the DOI: https://doi.org/10.7910/DVN/OL399P

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Figures

Figure 1 Monthly distribution of rhinovirus cases identified from surveillance of acute respiratory illness (ARIs) in children less than 60 months admitted to the Kilifi County Hospital, Kenya, 2007–2018. Also included on the secondary y-axis are the proportion (% positivity) of the samples from the in patients with ARI that were RV positive.

Figure 2 (A) Annual proportion of rhinovirus species across the 12-years period. (B) The total number (blue bars) and new number (purple bars) of RV types detected annually over the period 2007-2018. Also shown is the cumulative number of all the different RV types observed during the study period (black line). (C) The overall frequency of detection in months or the number of months each RV type was detected. The types are ordered alphabetically.

Figure 3 Quarterly proportions of RV types detected organised at the species level; shown here are the temporal trends of 5 most prevalent types per species while the rest are indicated as others. A) Quarterly proportion of RV A types (B) Quarterly proportion of RV B types, (C) Quarterly proportion of RV C types.

Figure 4 (A) Nucleotide variability across the sequenced VP4/VP2 region for types RV-A49 and RV-C2. For each type, the viruses were compared to the earliest sampled sequence. Vertical coloured bars show the nucleotide differences: red is a change to T, orange is a change to A, purple is a change to C and blue is a change to G. (B) Distribution of pairwise nucleotide difference for the VP4/VP2 region of types RV-A49 and C2.

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Figure 5 Bayesian phylogenetic trees showing the VP4/VP2 region of the RV types A49, C2, C38 and C11.Variant names are next to the phylogenetic clusters, e.g., v1 representing variant 1 for a specific type. Node support is indicated by (*) for posterior probabilities > 0.9.

Tables

Table 1 Number of different rhinovirus types identified in Kilifi, Kenya, 2007 -2018.

Table 2 Number of variants for the ten most prevalent RV types identified in Kilifi, Kenya, 2007 to 2018; * where b in a, b are the singletons (single sequence which genetically distinct from other types).

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Table 1 Number of different rhinovirus types identified in Kilifi, Kenya, 2007 -2018.

RV-A															
A49	A101	A12	A78	A56	A89	A20	A28	A40	A54	A1	A22	A61	A80	A29	A30
39	24	23	19	18	18	17	16	16	14	13	13	13	12	11	10
A58	A63	A82	A21	A75	A10	A47	A65	A106	A68	A103	A15	A43	A81	A88	A9
10	10	10	9	9	8	8	8	7	7	6	6	6	6	6	6
A16	A31	A46	A60	A66	A73	A104	A105	A13	A34	A36	A45	A55	A7	A90	A19
5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	3
A24	A32	A38	A39	A53	A8	A94	A96	A100	A102	A11	A23	A67	A18	A33	A41
3	3	3	3	3	3	3	3	2	2	2	2	2	1	1	1
A51	A59	A- untyped													
1	1	13													
RV-B															
B4	B70	B27	B42	B48	B86	B91	B104	B69	B102	B35	B72	B83	B101	B26	B6
7	5	4	4	4	4	4	3	3	2	2	2	2	1	1	1
B84	B92	B97													
1	1	1													

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RV-C															
C2	C38	C11	C43	C6	C21	C3	C10	C1	C14	C22	C40	C5	C27	C36	C25
29	26	26	26	22	21	20	18	17	16	16	16	16	15	13	12
C45	C37	C31	C32	C9	Cpat19	C46	Cpat18	C12	C16	C55	C15	C19	C41	C51	Cpat14
12	11	10	10	10	9	8	8	7	7	6	5	5	5	5	5
Cpat21	C42	Cpat20	C23	C33	C35	C39	C7	C8	C26	C44	C47	C49	Cpat17	Cpat22	Cpat28
5	4	4	3	3	3	3	3	3	2	2	2	2	2	2	2
C13	C17	C18	C24	C29	C30	C34	C48	C50	Cpat16	Cpat27	C-untyped				
1	1	1	1	1	1	1	1	1	1	1	11				

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Table 2 Number of variants for the ten most prevalent rhinovirus types identified in Kilifi, Kenya, 2007 to 2018

RV type	Number of sequences	Number of variants, singletons	Period (Year)
A12	23	9,5	v1(2007), v2(2007), v3(2009), v4(2009-2011), v5(2014), v6(2014), v7(2016), v8(2016), v9(2017)
A78	19	7,3	v1(2008), v2(2010-2011), v3(2011), v4(2013), v5(2013), v6(2018), v7(2018)
C2	29	12,5	v1(2007-2008), v2(2008), v3(2008), v4(2009), v5(2009), v6(2010), v7(2010), v8(2012), v9(2013), v10(2015), v11(2016-2017), v12(2017)
C11	25	8,3	v1(2007), v2(2008), v3(2008), v4(2008), v5(2009), v6(2010-2011), v7(2013), v8(2016)
C21	21	6,2	v1(2007), v2(2007-2012), v3(2010), v4(2012), v5(2015), v6(2018)
C38	26	6,0	v1(2007), v2(2007), v3(2007), v4(2009-2011), v5(2012) , v6(2016)
C3	20	7,2	v1(2009), v2(2010 -201), v3(2011), v4(2013), v5(2015), v6(2017),

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			v7(2018)
A49	39	11,4	v1(2008), v2(2008), v3(2008-2010), v4(2009), v5(2010-2011), v6(2012), v7(2012), v8(2014), v9(2015), v10(2014-2015), v11(2013-2014,2017)
A101	25	9,5	v1(2007), v2(2008), v3(2010), v4(2010), v5(2010-2013), v6(2015), v7(2013-2015), v8(2017), v9(2018)
C6	22	11,4	v1(2007), v2(2010 - 2011), v3(2010), v4(2010), v5(2010), v6(2011), v7(2011), v8(2011), v9(2013-2014), v10(2015), v11(2017)

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Figure 1

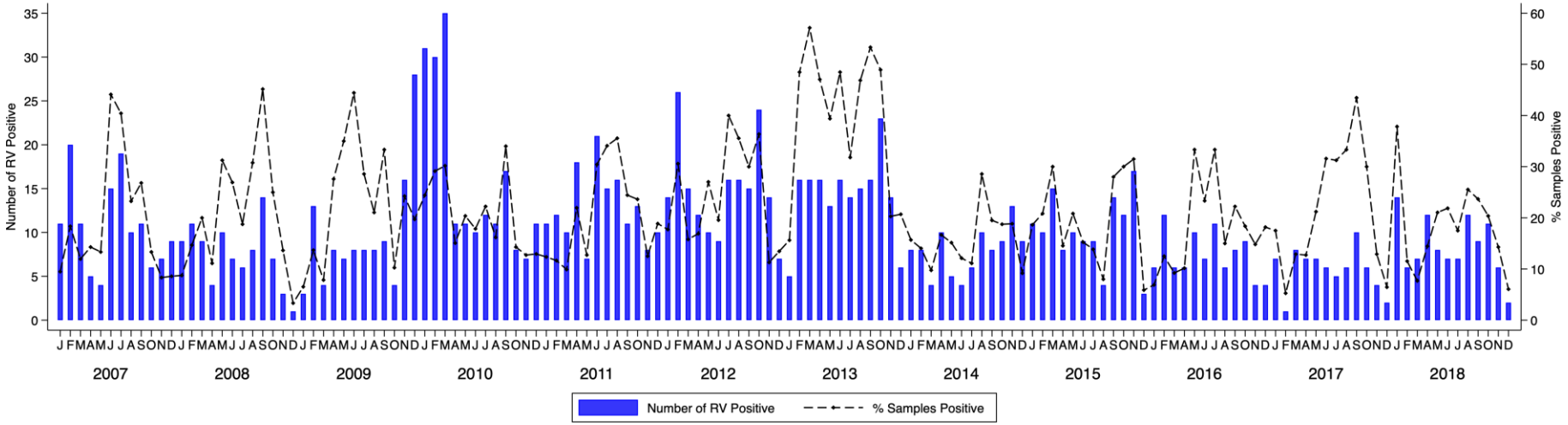

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Figure 2

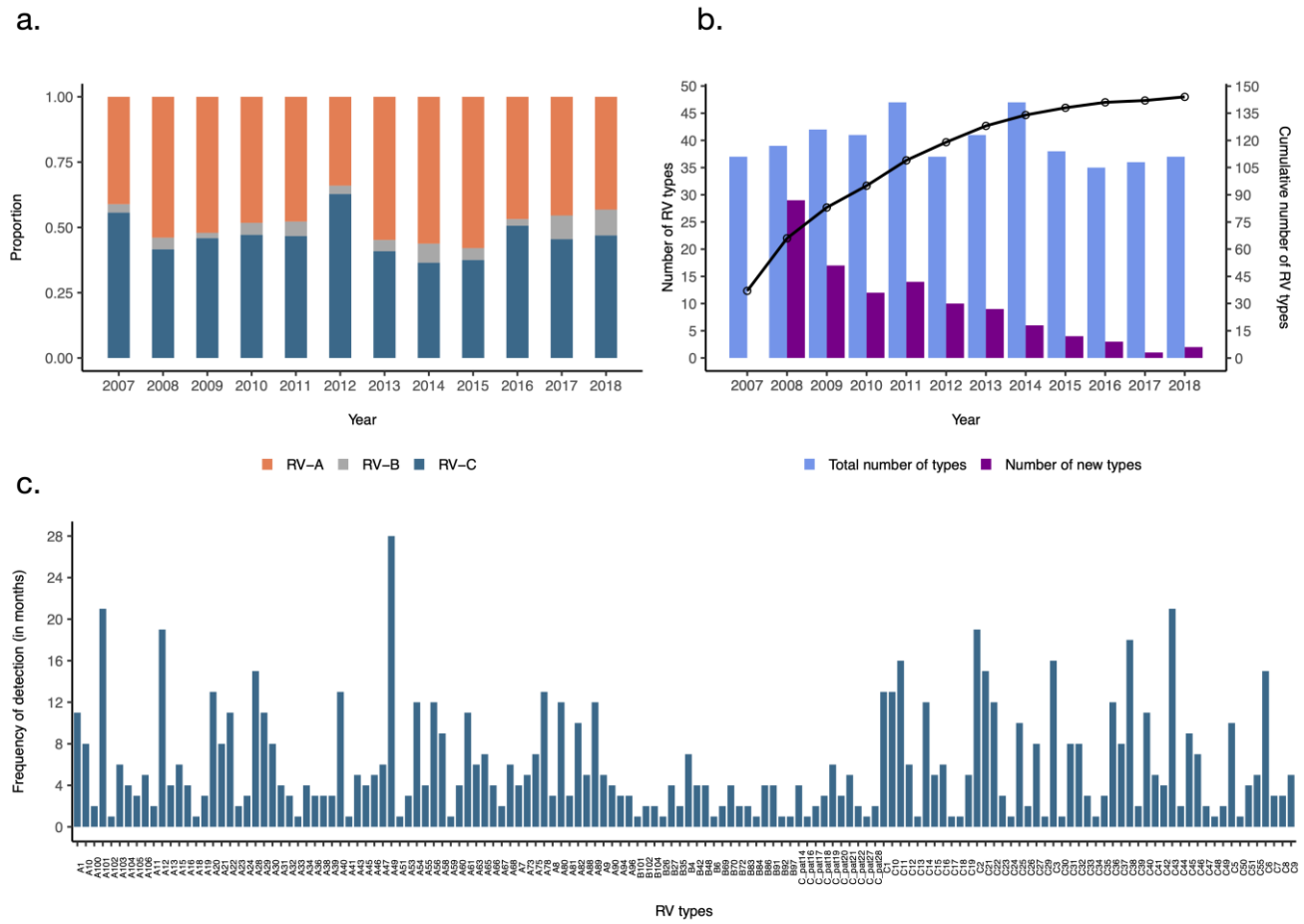

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Figure 3

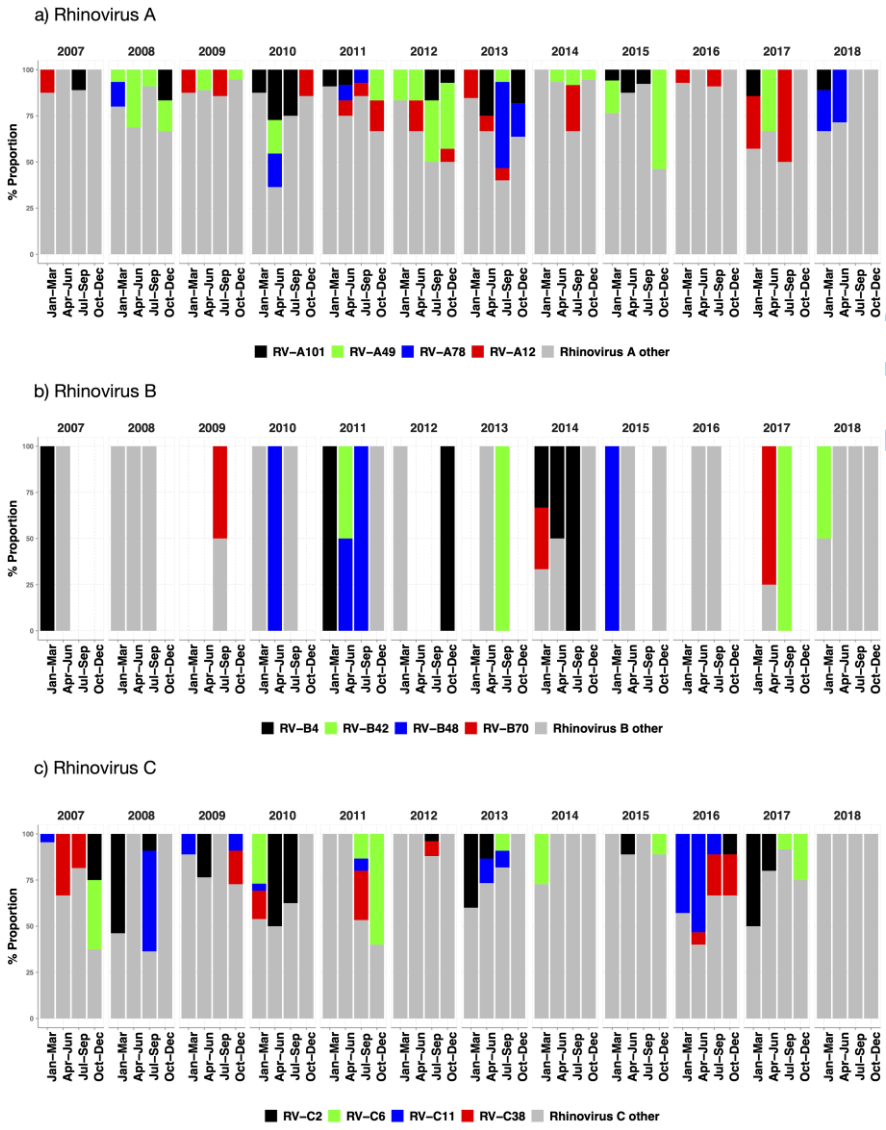

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Figure 4

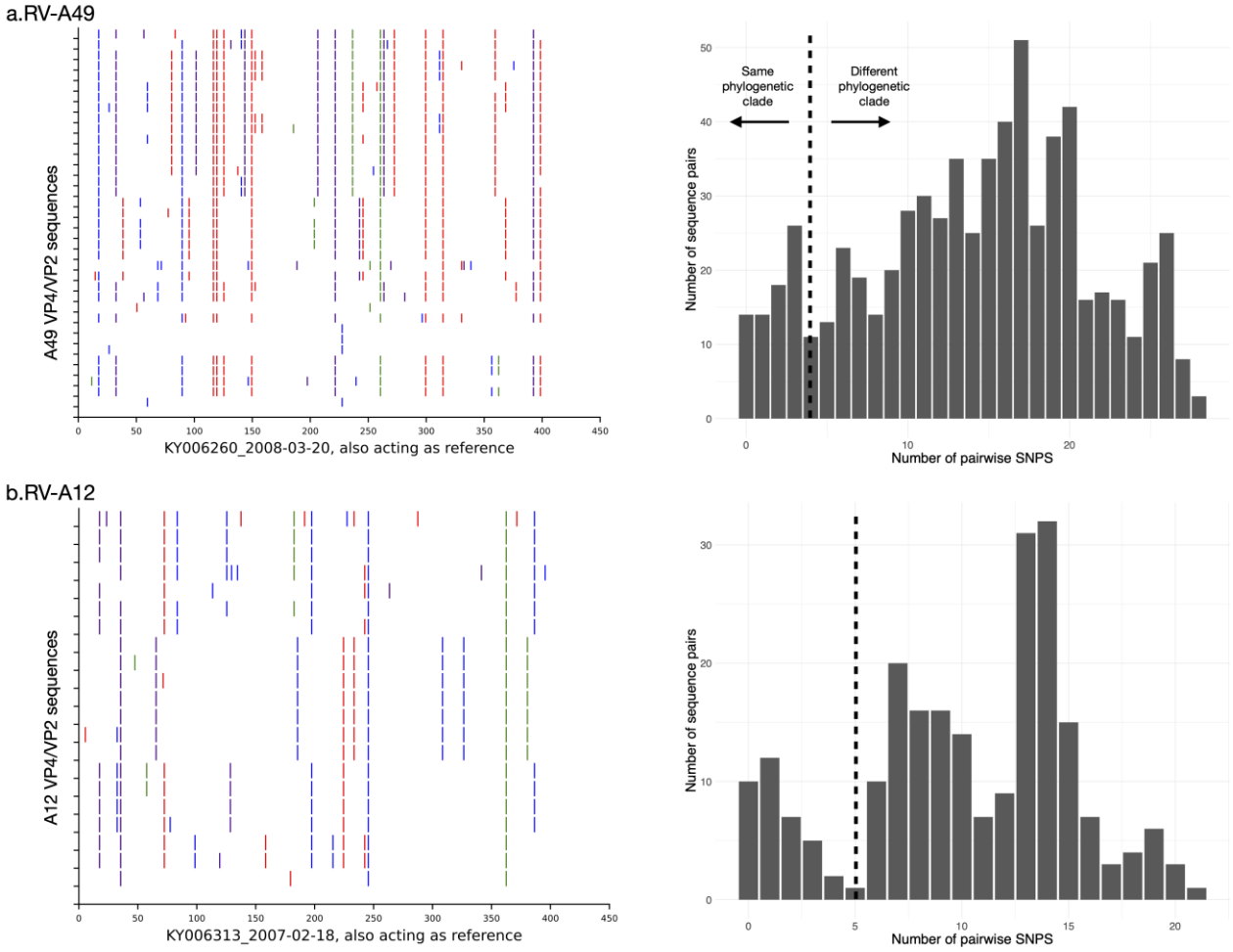

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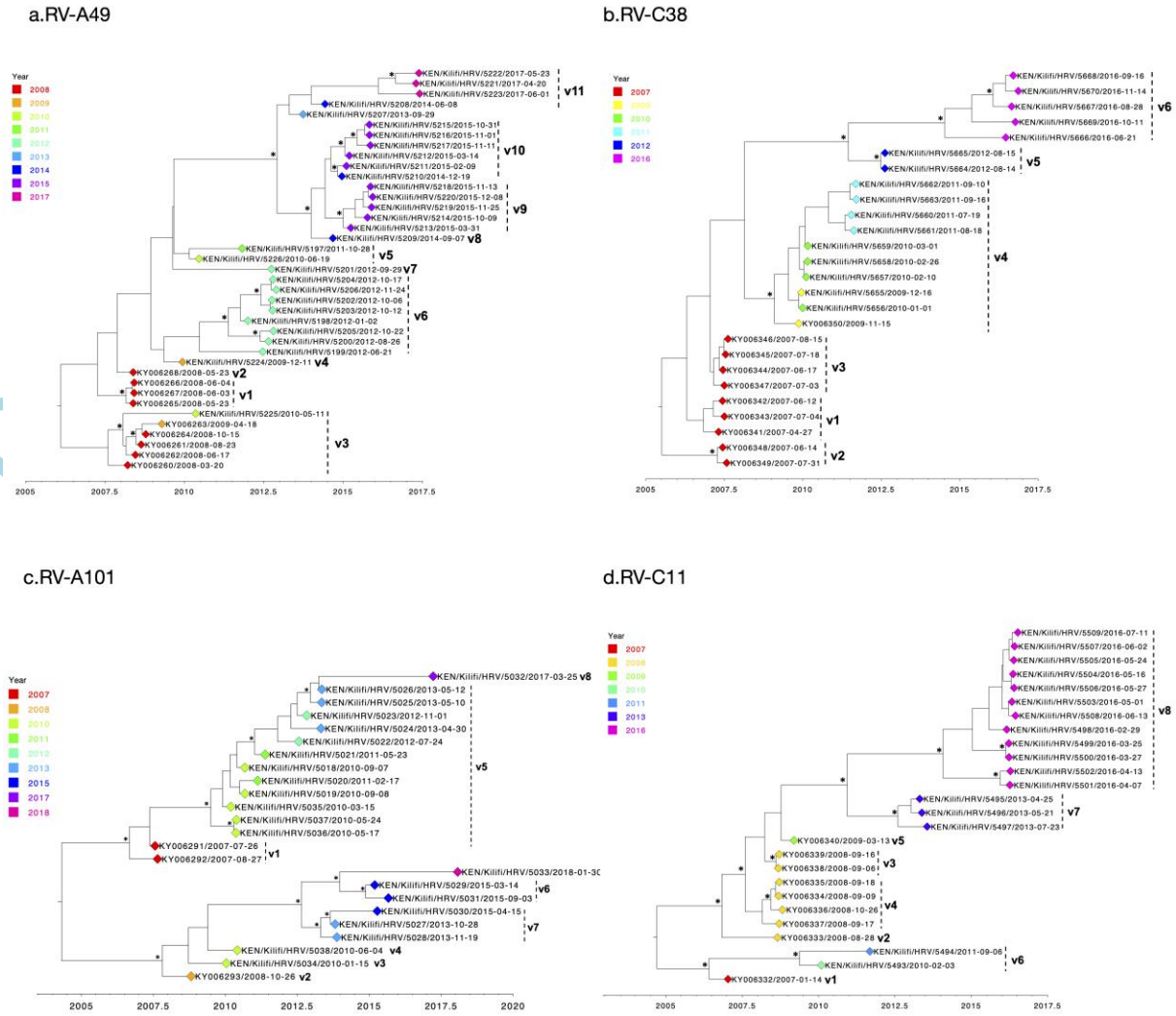
Figure 5



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