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Genomic analysis of coxsackieviruses A1, A19, A22, enteroviruses 113 and 104: viruses representing two clades with distinct tropism within enterovirus C

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Abstract

Coxsackieviruses (CV) A1, CV-A19 and CV-A22 have historically comprised a distinct phylogenetic clade within enterovirus C. Several novel serotypes that are genetically similar to these three viruses have been recently discovered and characterized. Here we report the coding sequence analysis of two genotypes of a previously uncharacterized serotype EV-C113 from Bangladesh and demonstrate that it is most similar to CV-A22 and EV-C116 within the capsid region. We sequenced novel genotypes of CV-A1, CV-A19, CV-A22 from Bangladesh and observed a high rate of recombination within this group. We also report genomic analysis of the rarely reported EV-C104 circulating in the Gambia in 2009. All available EV-C104 sequences display a high degree of similarity within the structural genes but form two clusters within the nonstructural genes. One cluster includes the recently reported EV-C117, suggesting an ancestral recombination between these two serotypes. Phylogenetic analysis of all available complete genome sequences indicates the existence of two subgroups within this distinct enterovirus C clade: one has been exclusively recovered from gastrointestinal samples, while the other cluster has been implicated in respiratory disease.

Introduction

The genus *Enterovirus* consists of twelve species, designated enterovirus (EV) A-J, and rhinovirus (HRV) A-C (http://www.picornaviridae.com). Of these, only EV-A,-B,-C,-D and HRV-A,-B,-C are associated with human infection and disease. Typically, an EV genome consists of a 7.3-7.5 kb-long, positive-sense, single-stranded RNA molecule with a single open reading frame (ORF). The ORF encodes a polyprotein that is organized into three regions, designated P1, P2 and P3. The P1 encodes VP4, VP2, VP3, and VP1 proteins that comprise the viral capsid. The P2 and P3 encode non-structural proteins involved in enzymatic activity and processing (2A, 2B, 2C and 3A, 3B, 3C, and 3D). Historically, serology was used to differentiate EV serotypes; more recently molecular methods are used with novel serotypes possessing <75% nucleotide (nt) and <85% amino acid (aa) identity within the VP1 relative to known EVs (Oberste *et al.* 1999a; Oberste *et al.* 1999b; Brown *et al.* 2009; Knowles *et al.* 2012). Over 120 EV serotypes have been reported, of which 23 are classified within the EV-C (http://www.picornaviridae.com). Phylogenetic analysis of early EV-C prototype strains revealed that it consists of two distinct subgroups; one includes Polio 1-3, coxsackieviruses (CV) A11, A13, A17, A20, A21, A24, and EV-C95, 96, 99, and 102; the other includes CV-A1, CV-A19, and CV-22 (Brown *et al.* 2003). The prototype strains of CV-A1, A19, and A22 are distinct from the former subgroup: they are monophyletic in P1-P3, and show little evidence for recombination with other EV-Cs. In addition, unlike most EVs that can be cultured in a wide variety of standard cell lines, the prototype strains of CV-A1, A19, and A22 have not been grown in cell culture (Schmidt *et al.* 1975; Lipson *et al.* 1988).

Although the prototype strains of CV-A1 (strain Tomkins, isolated in 1947), CV-A19 (8663, 1952), and CV–A22 (Chulman,1955) were reported in the 1950s (Brown *et al.* 2003; Knowles *et al.* 2012), these serotypes are only rarely identified during EV surveillance which has classically depended on growing the virus in cell culture (Khetsuriani *et al.* 2006). Although they have been implicated in enteric and neurological disease, little data exists on the genetic diversity, prevalence and spectrum of disease associated with infection (Begier *et al.* 2008; Kapusinszky *et al.* 2010; Chitambar *et al.* 2012; Tapparel *et al.* 2013). With more widespread implementation of molecular diagnostics, novel strains of these viruses have been discovered (Witso *et al.* 2006; Benschop *et al.* 2010; Oberste *et al.* 2013). Nonetheless, phylogenetic and genetic information remains limited. Full-length genome sequences are available for only four CV-A22, three CV-A1, and the prototype CV-A19 virus.

Recently, several novel serotypes genetically similar to CV-A1, CV-A19, and CV-A22 have been discovered using molecular methods. The first was EV-C104, discovered in Switzerland in patients with respiratory disease; thereafter, EV-C109 was identified in respiratory disease samples from Nicaragua (Tapparel *et al.* 2009; Yozwiak *et al.* 2010). In 2012, four additional serotypes (EV-C105, EV-C116, EV-C117, EV-C118) were reported and characterized; none were grown in cell culture (Daleno *et al.* 2012; Lukashev *et al.* 2012; Daleno *et al.* 2013; Tokarz *et al.* 2013). Analysis of phylogenetic and replication properties, including these novel serotypes, indicated three distinct groups recognized within EV-C (Lukashev *et al.* 2012). Each group makes up a distinct

93 phylogenetic cluster based on the P1 sequence. These groups also differ in 5' UTR,
94 growth in cell culture, and maintain reproductive isolation indicated by rare
95 recombination across other groups. The novel serotypes EV-C104, C105, C109, C117
96 and C118 represent group III, with an unconventional 5'UTR. Group II consists of CV-
97 A1, CV-A19, and CV-A22, as well as one novel serotype EV-C116 and are non-
98 cytopathogenic in RD cells. Group I is composed of the remaining serotypes, with a
99 conventional 5'UTR and cytopathogenicity in RD cell culture.
100
101 In surveys of samples from human subjects with respiratory and enteric disease, we
102 detected and identified EV-C serotypes from groups II and III. In an effort to shed light
103 on genetic relationships of these viruses, we sequenced genomes of several
104 representatives of these groups. In this report, we analyze genotypes of a new member of
105 group II, EV-C113, and novel genotypes of CV-A1, CV-A19, and CV-A22; all were
106 detected in stool samples from Bangladesh. We also describe strains of the rarely
107 reported EV-C104 circulating in the Gambia in 2009 and analyze the potential for
108 recombination and reproductive isolation within both viral groups.
109
110 **RESULTS**
111 *Characterization of EV-C113 as a member of group II of EV-C*
112 Of 149 stool samples from Bangladesh, 52 were positive for at least one EV; 4 samples
113 represented mixed infections. An EV-C virus was identified in 19 samples of which 13
114 belonged to group II and none to group III. Viruses identified in three samples exhibited
115 low nucleotide (nt) homology (<80%) to CV-A1, CV-A19 and CV-A22 within the VP4/2

116 region suggesting they may represent a unique EV serotype. Amplification and sequence
117 analysis of the VP1 gene indicated that these viruses were approximately 90% identical
118 to 324 nt VP1 sequences obtained from stool samples from Bolivia (GenBank accession
119 number JX219564) that included a virus designated EV-C113
120 (http://www.picornaviridae.com). The viruses closest to EV-C113 were EV-C116 and
121 CV-A22, and placed this uncharacterized serotype along with CV-A1 and CV-A19
122 within group II of EV-C. To compare the complete phylogenetic relationship of EV-C113
123 to other viruses within this clade, we obtained the full-length coding sequence of EV-
124 C113 viruses from two samples, designated BBD48 and BBD83 (GenBank accession
125 numbers KC344833 and KC344834) (Table 1). The third EV-C113-positive sample
126 contained the same genotype as BBD48 (99% nt identity within the VP4/2) and was not
127 analyzed further. BBD48 and BBD83, obtained from samples in 2006 and 2009,
128 respectively, represented two genotypes of EV-C113; the 6618 nt ORFs of the two
129 viruses were 91.7% identical to each other (99.1% aa identity). Within the P1 region, the
130 two viruses had 93.3% nt identity (99.6 aa identity). The complete 888 nt of VP1
131 sequence of EV-C113 BBD83 was 64% similar on a nt level and 81% similar on the aa
132 level relative to the nearest serotype, CV-A22 ban99-10427, also from Bangladesh
133 (DQ995647).
134
135 The stool sample with EV-C113 BBD66 also contained a CV-A1 virus. We detected CV-
136 A1 in three other samples: one of these contained an EV-B virus. CV-A22 was present in
137 two samples: one of them also contained an EV-B virus. Two distinct genotypes of CV-
138 A19 were detected amongst four samples. We sequenced the complete coding sequence

139 of both genotypes, designated as strains BBD26 and BBD66. Complete coding sequence
140 was also obtained for CV-A1 (one sample: BBD34) and CV-A22 viruses (two samples:
141 BBD1 and BBD58) (Table 1).
142 Within the P1 region, CV-A19 viruses formed a distinct phylogenetic branch from the
143 other four serotypes of group II and exhibited the highest degree of aa conservation in
144 relation to their prototype strain (Fig. 1(a)). Throughout the P1 region, the prototype
145 strain had 82.4% nt identity (98.1% aa identity) to BBD26 and 83.4% nt identity (99.0%
146 aa) to BBD66; BBD26 and BBD66 had 85.1% nt identity (98.3% aa identity) to each
147 other. CV-A1 BBD34 had <85% nt identity (<93% aa identity) to the three other CV-A1
148 genomes. CV-A22 BBD01 and CV-A22 BBD58 were both obtained from samples
149 collected in 2009 and had 94.1% nt identity (96.5% aa identity) to each other. Both were
150 most similar to the CV-A22 detected in 1999 in Bangladesh. BBD01 was nearly identical
151 on the aa level (99.1%); BBD58 had 96.2% aa identity. Overall, among the 6 full
152 genomes of CV-A22, the P1 aa identity among strains ranged from 99.1% to 86.2%.
153
154 While all the new viruses analyzed here clustered with their prototype strains within the
155 P1, none did so in P2 or P3, suggesting these viruses have undergone a number of
156 recombination events (Fig. 1(b) and 1(c)). Both EV-C113 strains cluster together within
157 the P1 and P2, but BBD83 from 2009 clusters with CV-A22 BBD01 from 2009 while
158 BBD48 from 2006 clusters with CV-A22 from 1999. In both P2 and P3, CV-A1 BBD34
159 clusters with CV-A22 BBD58; both CV-A1 BBD34 and CV-A22 BBD58 came from
160 samples collected in 2009. Interestingly, unlike the other serotypes, the two new CV-A19
161 genotypes clustered together within both P2 and P3 regions which suggests at least some

selective reproductive isolation for this serotype. Overall, despite the apparent recombination, the P3 aa sequence of all five serotypes was highly conserved, with all viruses being within 97.9% identical to each other.

Analysis of EV-C104 from the Gambia

EV-C104 is a rare EV serotype; thus far it was only reported from cases of respiratory disease in Switzerland, Italy and Japan (Tapparel *et al.* 2009; Kaida *et al.* 2012; Piralla *et al.* 2012). During a case-control study of pediatric respiratory disease in the Gambia, we detected EV-C104 in 5 out of 819 samples. The five samples, designated GAM693, GAM714, GAM724, GAM735 and GAM738 were obtained between March 26 and June 2 of 2009, all from individuals residing within the Greater Banjul area (Table 2). Three samples came from children with pneumonia, while two EV-C104-positive samples were identified among control children recruited from the community. To more closely characterize the strains circulating in the Gambia, we sequenced the complete coding sequence of GAM693 and GAM714, as well as partial genome fragments from the remaining three samples. The complete VP1 sequences of the GAM strains had a nucleotide divergence of 3.8% to 1.1 % (99-100% aa identity). Analysis of all VP1 sequences currently available revealed that GAM693 clustered with strains reported from Europe between 2005-2009, while the remaining four viral sequences from the Gambia clustered with the strain recently described from Japan in 2011 (Fig. 2(a)). Analysis of the P1-P3 regions from all available EV-C104 genomes revealed high similarity within the P1, with >5% nt divergence among all identified strains (Fig. 1(a)). Within the P2 and P3 regions, all the GAM strains clustered with the strains from Japan and Italy, but

were significantly divergent from the prototype strain described in Switzerland (nt divergence: 6% within the P2, 17% within the P3) (Fig. 1(b) and 1(c)). Only a single complete genome is available from EV-C104 identified in Switzerland, but several 3Dpol partial sequences exist and were used for phylogenetic analysis. The analysis revealed two clusters; all EV-C104 strains from Switzerland detected in samples originating in 2005 clustered together, while a second cluster consisted of all strains recovered in 2007 and beyond (single strain from Switzerland from 2007; all Italian (2009); Gambian (2009) strains; 2011 Japanese strain) (Fig. 2 (b)). Additionally, the phylogenetic tree of P3 from all EV-C serotypes from groups II and III indicated that strains from the Gambia, Italy and Japan cluster with the recently described EV-C117 serotype rather than the prototype EV-C104 from Switzerland. This suggests that a possible recombination may have occurred between ancestral EV-C104 strains and an EV-C117-like virus. Bootscan analysis did reveal a probable recombination event between EV-C104 and EV-C117 around position 4750 within the P2 region, which mapped to an area within the 2C gene (Fig. 3).

Analysis of complete genomes indicated that viruses in group III form two distinct clusters within both P2 and P3 regions (Fig. 1(b) and 1(c)). EV-C104 and EV-C117 form one cluster, while EV-C105, EV-C109, EV-C118 form the second, more divergent cluster. In P3, the viruses in each cluster diverge by >30% nt and >13% aa from viruses in the other cluster.

Incidence and tropism of viruses in groups II and III

208 Analysis of the literature and available sequence data reveals an overall low incidence of
209 group III viruses. EV-C104 was the lone group III virus detected in the samples from the
210 Gambia. We also did not detect any group III viruses in 1003 NPA samples from South
211 Africa obtained from 2000-2001 or from 940 NPA samples from New York City in 2009-
212 2010 (Tokarz *et al.* 2011; Tokarz *et al.* 2012). Only single strains of EV-C105 and EV-
213 C118 were detected within 163 respiratory samples from Peru (Tokarz *et al.* 2013). We
214 did not detect group II viruses in any of these sample sets. In addition, group II viruses
215 have not been identified in any prior surveys of respiratory disease where molecular EV
216 typing was performed and have not been linked with respiratory disease (Piralla *et al.*
217 2012; Xiang *et al.* 2012; Tapparel *et al.* 2013). Analysis of sample origin for all VP1
218 sequences from group II and III of EV-C (deposited in GenBank as of January 15, 2013),
219 indicates that every sequence for a group II virus was obtained from stool samples and
220 none from the respiratory tract (Supplemental table 1). Clinical status varied widely.
221 Some subjects were asymptomatic whereas others had GI disease or flaccid paralysis.
222 Conversely, viruses from group III have predominately been detected in nasal swabs or
223 nasopharyngeal aspirates from subjects who were asymptomatic or had respiratory
224 disease. The lone exception is one EV-C105 strain detected in stools of a patient with
225 flaccid paralysis from the Democratic Republic of Congo (Lukashev *et al.* 2012).
226
227 **Discussion**
228 CV-A1, CV-A19 and CV-A22 have historically comprised a distinct clade within EV-C
229 based on their phylogeny, lack of evidence for recombination, and inability to grow in
230 tissue culture (Brown *et al.* 2003). With the characterization of EV-C113, ten serotypes

231 are now recognized within this group. Initially, Lukashev et al divided these viruses into
232 two subgroups based on cytopathic effect (or lack thereof), reproductive isolation, and the
233 presence of a 5'UTR sequence distinct with respect to other EV-C viruses (Lukashev *et*
234 *al.* 2012). Our results confirm this analysis and indicate that the two subgroups also differ
235 in tropism and clinical outcome. Group II viruses typically replicate in the
236 gastrointestinal tract are most frequently implicated with gastroenteritis and herpangina;
237 CV-A19 and CV-A22 have also been linked to aseptic meningitis (Tapparel *et al.* 2013).
238 In contrast, group III viruses are more commonly associated with respiratory disease and
239 all but a single virus have been detected in nasal or nasopharyngeal samples. Both groups
240 have rarely been reported due to the fact that EV surveillance historically relied on
241 viruses being isolated in culture. Ideally, surveillance and molecular typing of viruses in
242 both respiratory and stool samples from the same geographical area would determine the
243 prevalence and confirm the tropism of these viruses.
244
245 Differential tropism among viruses within the same EV species has been documented.
246 Within EV-D, enterovirus 68 (EV-D68) has been associated with respiratory disease,
247 while enterovirus 70 is primarily linked to acute hemorrhagic conjunctivitis (Schieble *et*
248 *al.* 1967; Mirkovic *et al.* 1973). A number of factors can contribute to differential
249 tropisms amongst EVs. One of the main determinants is the receptor or co-receptor used
250 for cellular entry. Several EV-C serotypes, such as CV-A21, use intercellular adhesion
251 molecule 1 (ICAM-1) as a cellular receptor and are mainly associated with mild
252 respiratory disease (Xiao *et al.* 2001). ICAM-1 is also utilized by major group
253 rhinoviruses, which are predominately found in the respiratory tract and associated with

254 respiratory disease (Greve *et al.* 1989). Utilization of different receptors may also have a
255 role in tropism of group II and III viruses.
256
257 Another explanation for the paucity of group III viruses in gastrointestinal samples might
258 be acid lability as is the case for rhinoviruses and EV-D68. Although acid sensitivity
259 would restrict group III viruses to the respiratory tract, a single representative of this
260 group has been detected in a stool sample (Lukashev *et al.* 2012). This is not unusual,
261 however, as rhinoviruses can be detected in stool samples (Harvala *et al.* 2012; Oberste *et*
262 *al.* 2013). In the stool samples from Bangladesh, we identified seven samples containing
263 rhinoviruses. We cannot experimentally test the acid lability hypothesis because group II
264 and III viruses have historically not been propagated on culture. The recent discovery of
265 CV-A1 stains capable of growth in RD cell lines, marks a potential breakthrough in
266 studying group II and III viruses, which would be an important first step for elucidating
267 components of the life-cycle of these viruses (Sun *et al.* 2012).
268
269 Beginning with EV-C104 in 2009, five novel serotypes of group III have been reported at
270 the time of writing. While clearly displaying an association with respiratory disease, in
271 most cases these viruses were identified not in large clusters but in rare individual cases
272 within a large cohort. Rarity of detection, in combination with difficulties in propagating
273 these viruses in culture, limits our ability to investigate their biology and genetic
274 diversity. From a phylogenetic and clinical perspective, EV-C104 is the best
275 characterized virus in group III. In addition to the three cases described in this report,
276 only 16 other probable cases have been documented. EV-C104 was found in 8 patients in

277 Switzerland with otitis media or pneumonia and in 7 patients in Italy with upper
278 respiratory tract (4 cases, 2 immumocompromised), or with lower respiratory tract (3
279 cases, two immunocompromised) disease (Tapparel *et al.* 2009; Piralla *et al.* 2012). A
280 single case of upper respiratory tract infection was reported from Japan (Kaida *et al.*
281 2012). Based on available clinical data, EV-C104 infection ranges from asymptomatic to
282 pneumonia, although the majority of cases appear to result in mild respiratory disease.
283 All three symptomatic cases from the Gambia presented with pneumonia; however, we
284 cannot rule out that EV-C104 may have represented a secondary infection as we did not
285 observe a difference in viral load between clinical cases and controls. No additional viral
286 agents were found in 2 of the 3 cases; nonetheless, a primary or secondary bacterial
287 infection cannot be excluded.
288
289 Genetic diversity in enteroviruses is generated by genetic drift and recombination. Both
290 inter- and intratypic recombination occurs frequently (Santti *et al.* 1999; Brown *et al.*
291 2003; Oberste *et al.* 2004; Oberste *et al.* 2004; Simmonds *et al.* 2006; Bessaud *et al.*
292 2011; Combelas *et al.* 2011). The majority of recombination occurs within P2 and P3
293 regions, with little or no recombination within the P1. Although molecular typing using
294 sequence divergence within VP1 is commonly used for enterovirus identification, such
295 assays limit the phylogenetic and biological information generated by analyzing complete
296 genomes (Lukashev 2005). Recombination can alter a virus by conferring properties of
297 another virus that may offer selective advantages and increase fitness. One of the criteria
298 for demarcation of the three EV-C groups was reproductive isolation, due to very rare
299 recombination among viruses in different groups. Thus far, only a single serotype, EV-

C96, had shown signs of recombination between groups I and II (Smura *et al.* 2007; Brown *et al.* 2009). It is apparent that viruses in group II undergo frequent recombination similar to viruses in group I. Recently, a CV-A22 from Hong Kong and CV-A1 from China both were shown to have undergone recombination with other group II viruses (Yip *et al.* 2011; Sun *et al.* 2012). Our results show that amongst the group II viruses from Bangladesh there were many rearrangements within the P2 and P3 indicative of recombination although it was also restricted among group II viruses with no evidence of recombination with other groups. Within viruses of group III, recombination was previously proposed but has not been conclusively shown (Lukashev *et al.* 2012; Tokarz *et al.* 2013). Analysis of the P3 region of EV-C104 indicates that this virus has undergone a rearrangement within the last decade, likely due to recombination with a EV-C117-like virus. While this is the first example of recombination among viruses in group III, we expect with the availability of more full-length genomes more frequent evidence of recombination will be observed. Such studies will allow for a more thorough phylogenetic analysis of both groups of EV-C viruses.

Materials and Methods.

Stool samples (n=149) obtained from children <5 years old with gastrointestinal disease in Bangladesh were screened for EVs using primers 5'Fwd-TCCTCCGGCCCCTGAATGCGGCTAATCC and 5'Rev-GAAACACGGWCACCAAAGTASTCG. EV-positive samples were typed using PCR assays targeting the VP4/2, and 5'UTR of EVs (Tokarz *et al.* 2012; Tokarz *et al.* 2013). The complete coding sequences of selected viruses were obtained by consensus PCR.

Full genomes of group II and III viruses were aligned, and multiple consensus primers were designed (supplemental table 2). Overlapping PCR products were generated and used to resolve full-length sequences. In cases where multiple viruses were present in the same sample, PCR products were cloned and multiple fragments sequenced to obtain specific virus sequence.

Samples with EV-C104 were obtained during the course of a case-control study of pediatric respiratory disease in the Gambia. All samples were collected between 2007 and 2009 and screened for respiratory pathogens using MassTag PCR (Briese *et al.* 2005). EV-positive samples were typed as above; EV-C104 sequence was amplified by consensus PCR using EV-C104 genomes AK11 and CL-1 as reference (AB686524 and EU840733).

Alignments, phylogenetic trees, and distance matrices were obtained with Mega5 software (Tamura K 2007). Phylogenetic trees were generated using the maximum-likelihood method with 1000 bootstrap replicates. Similarity plots and recombination analysis were performed using SimPlot 3.5 with manual bootscaning using the Kimura distance model with a 200 nt-window and a step size of 20nt.

All sequences were deposited in GenBank under accession numbers KC344833-KC344834 and KC785523-KC785535.

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348

349 Table 1. Group II viruses analyzed in this study.

Sample	Serotype	Origin	Date of collection	Sample type
BBD48	EV-C113	Bangladesh	7/2006	stool
BBD83	EV-C113	Bangladesh	11/2009	stool
BBD01	CV-A22	Bangladesh	1/2009	stool
BBD58	CV-A22	Bangladesh	6/2009	stool
BBD34	CV-A1	Bangladesh	4/2009	stool
BBD26	CV-A19	Bangladesh	3/2009	stool
BBD66	CV-A19	Bangladesh	10/2006	stool

350

351

352 Table 2. EV-C104-positive samples from the Gambia

Strain	**Origin**	**Date of collection**	**Sample source**	**Clinical outcome**	**Temp (C)**	**Viral load ^**	**Age (months)**	**Sex**
GAM693	The Gambia	3/26/2009	NPA	pneumonia	36.5	1.7 X 10e5	25	M
GAM714	The Gambia	4/28/2009	NPA	control	-	4.3 X 10e5	25	F
GAM724	The Gambia	5/12/2009	NPA	control	-	3.6 X 10e3	7	F
GAM735	The Gambia	6/1/2009	NPA	pneumonia	38.8	8.3 X 10e3	19	M
GAM738*	The Gambia	6/2/2009	NPA	pneumonia	40.0	N/A	15	F

353

354 *Sample also contained rhinovirus C.

355 ^ represents copy number/100ul of sample

356 NPA - nasopharyngeal aspirate; N/A – not available

Figure legends

FIGURE 1. Maximum-likelihood phylogenetic tree constructed from all available complete P1 (A) P2 (B) and P3 (C) sequences of group II (non-respiratory tropic) and group III (respiratory tropic) viruses. For each virus, the accession number, serotype and strain name are provided. * indicate sequences identified for this study; # indicates the lone group III sequence obtained from a stool sample. 1C contains P3 sequences from recombinant EV-C96 which is not part of either group.

FIGURE 2. Analysis of EV-C104 from the Gambia. A) maximum-likelihood phylogenetic constructed on A) of the complete VP1 sequence B) a 398 base pair fragment of all available sequences within the 3Dpol gene. For the 3Dpol tree, the year of collection is provided next to the accession numbers, serotype and strain designation.

FIGURE 3.

Similarity plot (A) and bootscan plot (B) of EV-C104 GAM714 compared to other group III viruses.

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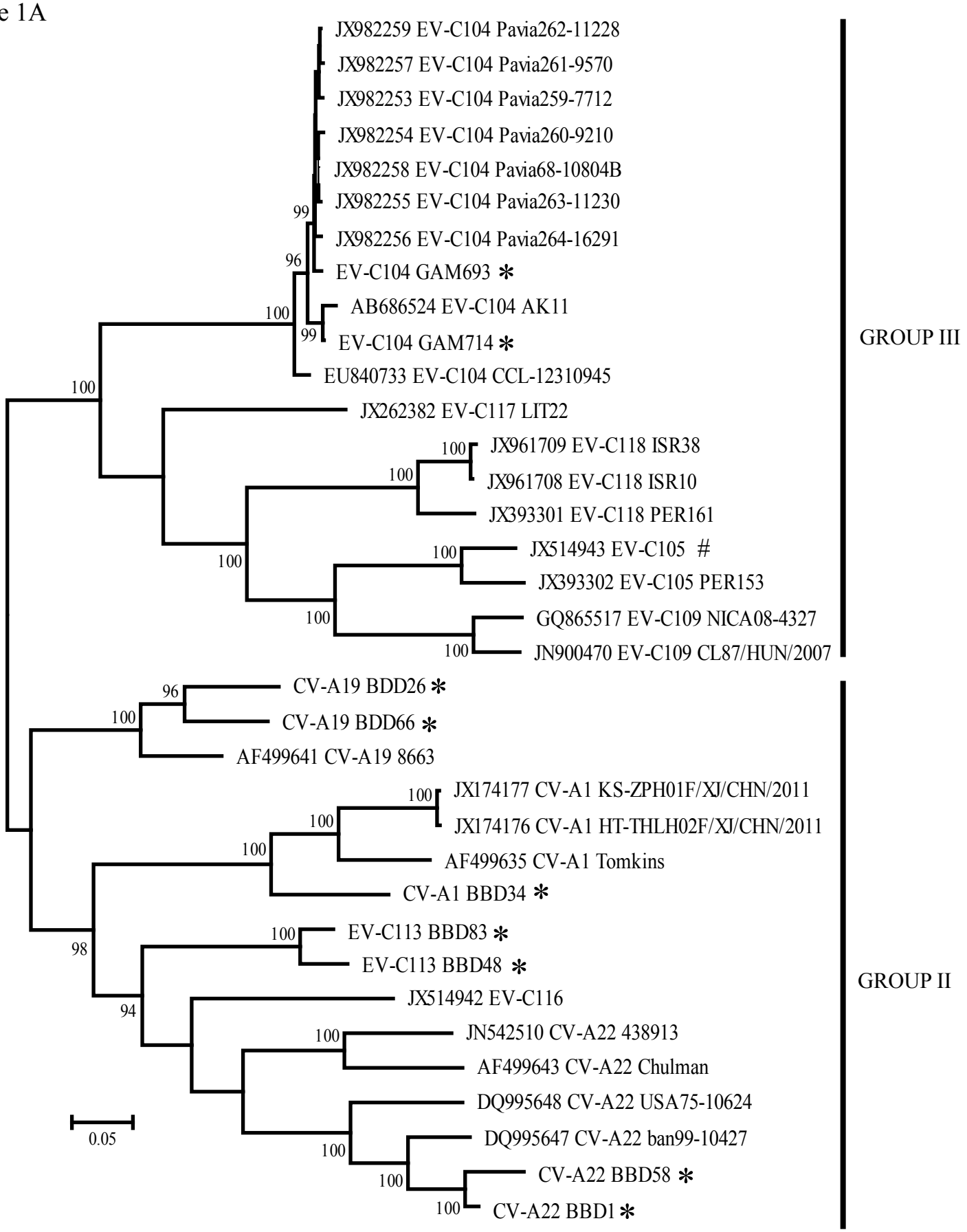
Figure 1A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JX982255 EV-C104 Pavia263-11230
JX982256 EV-C104 Pavia264-16291
EV-C104 GAM693 *
AB686524 EV-C104 AK11
EV-C104 GAM714 *
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JX961709 EV-C118 ISR38
JX961708 EV-C118 ISR10
JX393301 EV-C118 PER161
JX514943 EV-C105 #
JX393302 EV-C105 PER153
GQ865517 EV-C109 NICA08-4327
JN900470 EV-C109 CL87/HUN/2007
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CV-A19 BDD66 *
AF499641 CV-A19 8663
JX174177 CV-A1 KS-ZPH01F/XJ/CHN/2011
JX174176 CV-A1 HT-THLH02F/XJ/CHN/2011
AF499635 CV-A1 Tomkins
CV-A1 BBD34 *
EV-C113 BBD83 *
EV-C113 BBD48 *
JX514942 EV-C116
JN542510 CV-A22 438913
AF499643 CV-A22 Chulman
DQ995648 CV-A22 USA75-10624
DQ995647 CV-A22 ban99-10427
CV-A22 BBD58 *
CV-A22 BBD1 *
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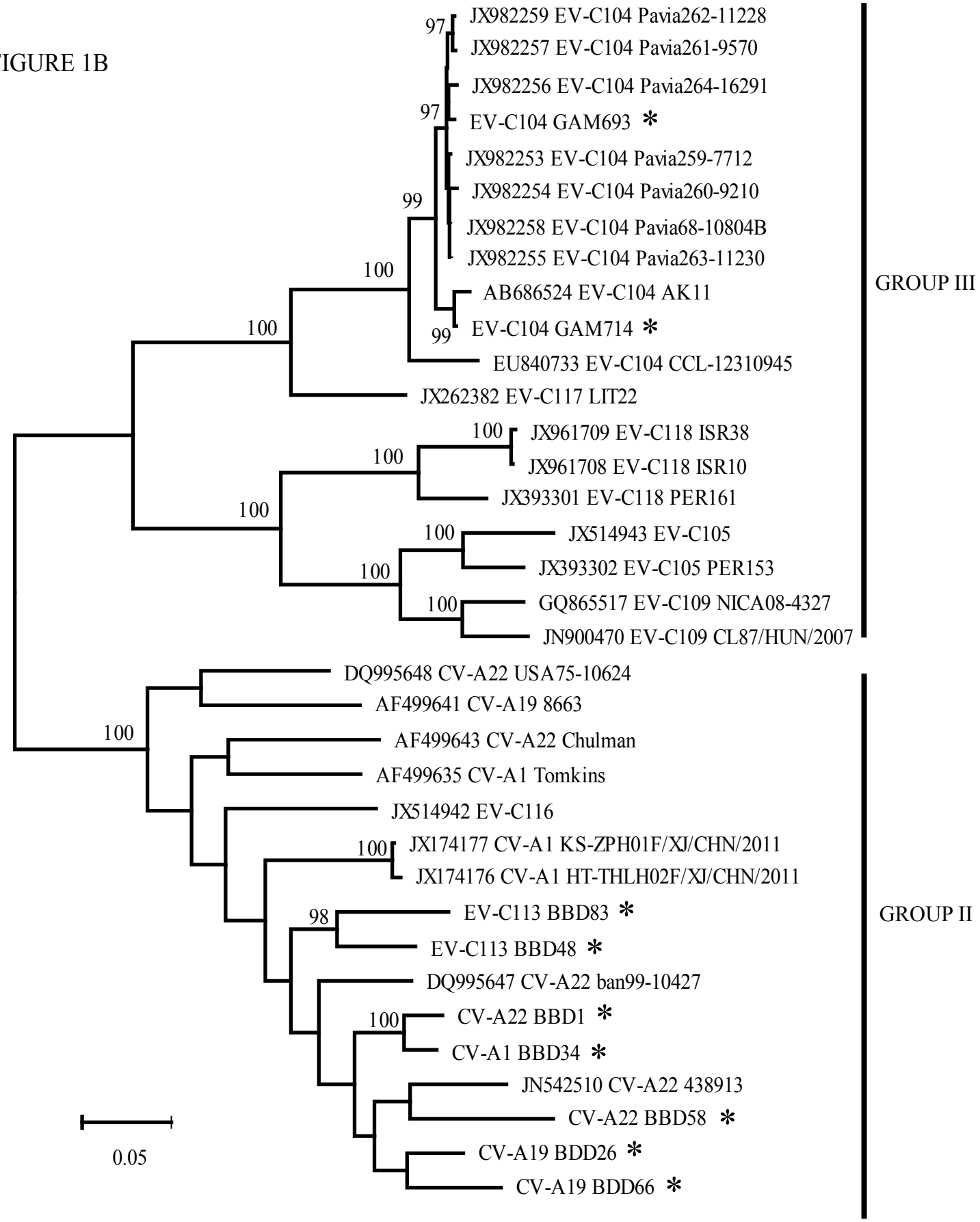
100
0.05
GROUP III
GROUP II

FIGURE 1B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JX982254 EV-C104 Pavia260-9210
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EV-C104 GAM714 *
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JX393302 EV-C105 PER153
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JN900470 EV-C109 CL87/HUN/2007
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AF499641 CV-A19 8663
AF499643 CV-A22 Chulman
AF499635 CV-A1 Tomkins
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EV-C113 BBD83 *
EV-C113 BBD48 *
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CV-A22 BBD1 *
CV-A1 BBD34 *
JN542510 CV-A22 438913
CV-A22 BBD58 *
CV-A19 BDD26 *
CV-A19 BDD66 *
97
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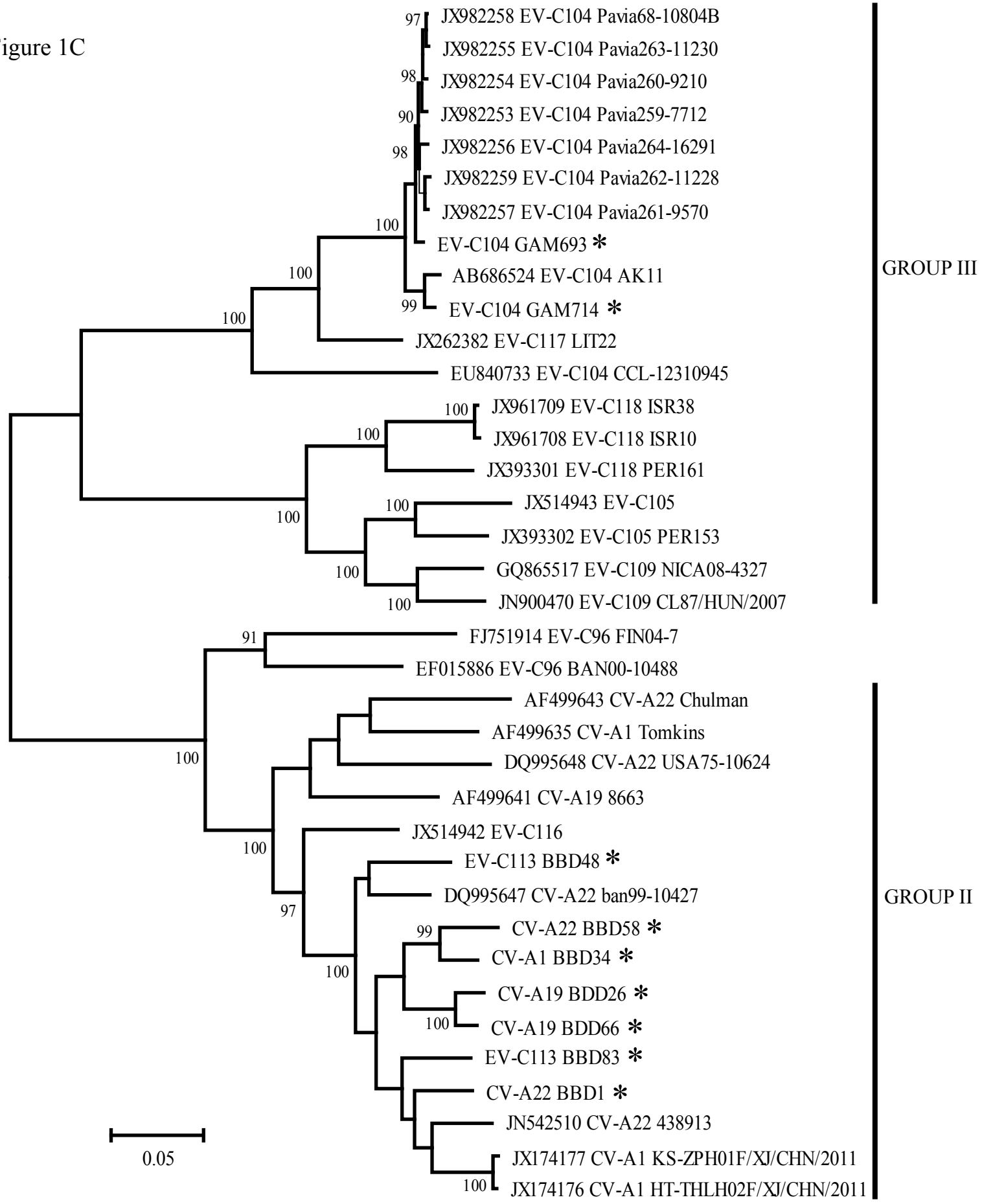
GROUP III
GROUP II

Figure 1C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JX982256 EV-C104 Pavia264-16291
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EV-C104 GAM693 *
AB686524 EV-C104 AK11
EV-C104 GAM714 *
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EU840733 EV-C104 CCL-12310945
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AF499635 CV-A1 Tomkins
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DQ995647 CV-A22 ban99-10427
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EV-C113 BBD83 *
CV-A22 BBD1 *
JN542510 CV-A22 438913
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GROUP III
GROUP II
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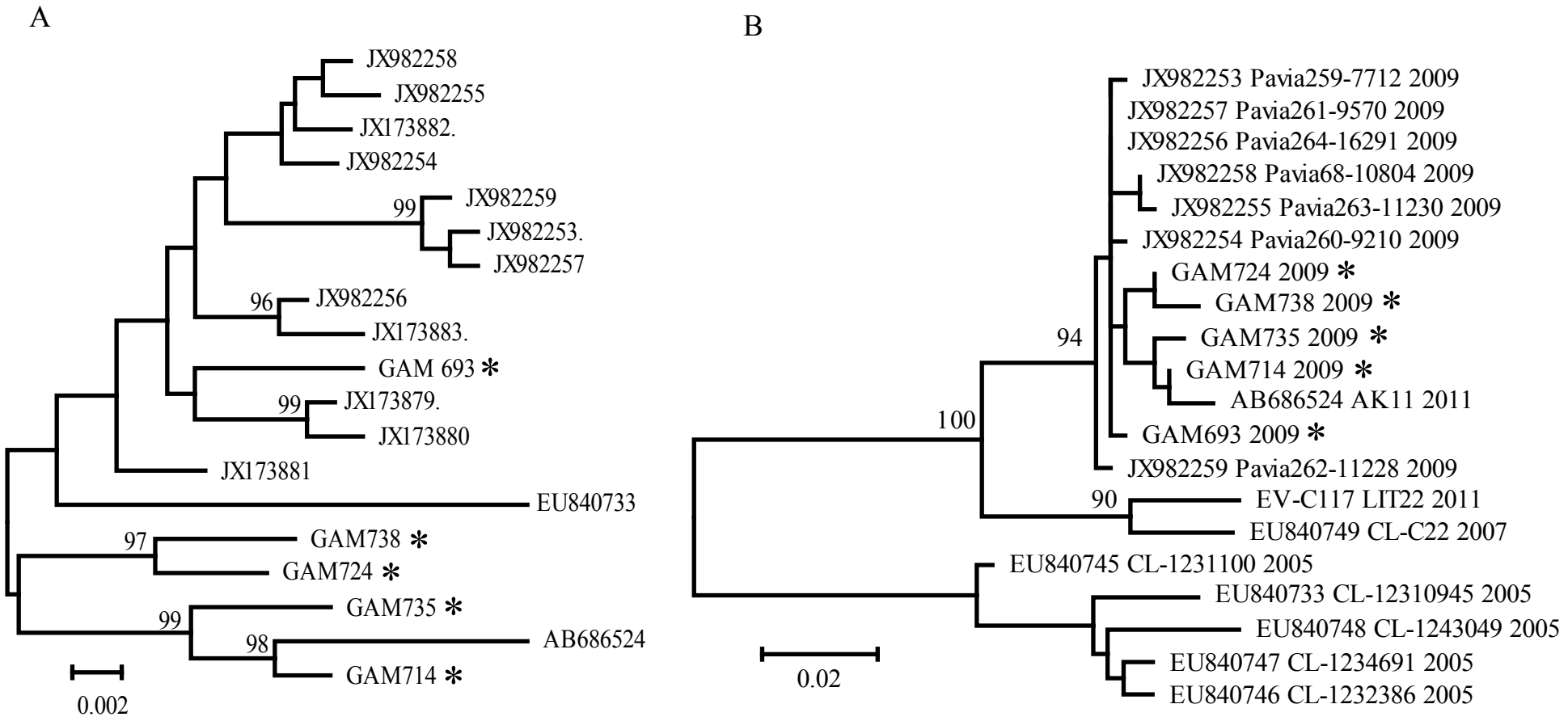
FIGURE 2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JX173883.
GAM 693 *
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JX173881
EU840733
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GAM735 *
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AB686524
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JX982253 Pavia259-7712 2009
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EV-C117 LIT22 2011
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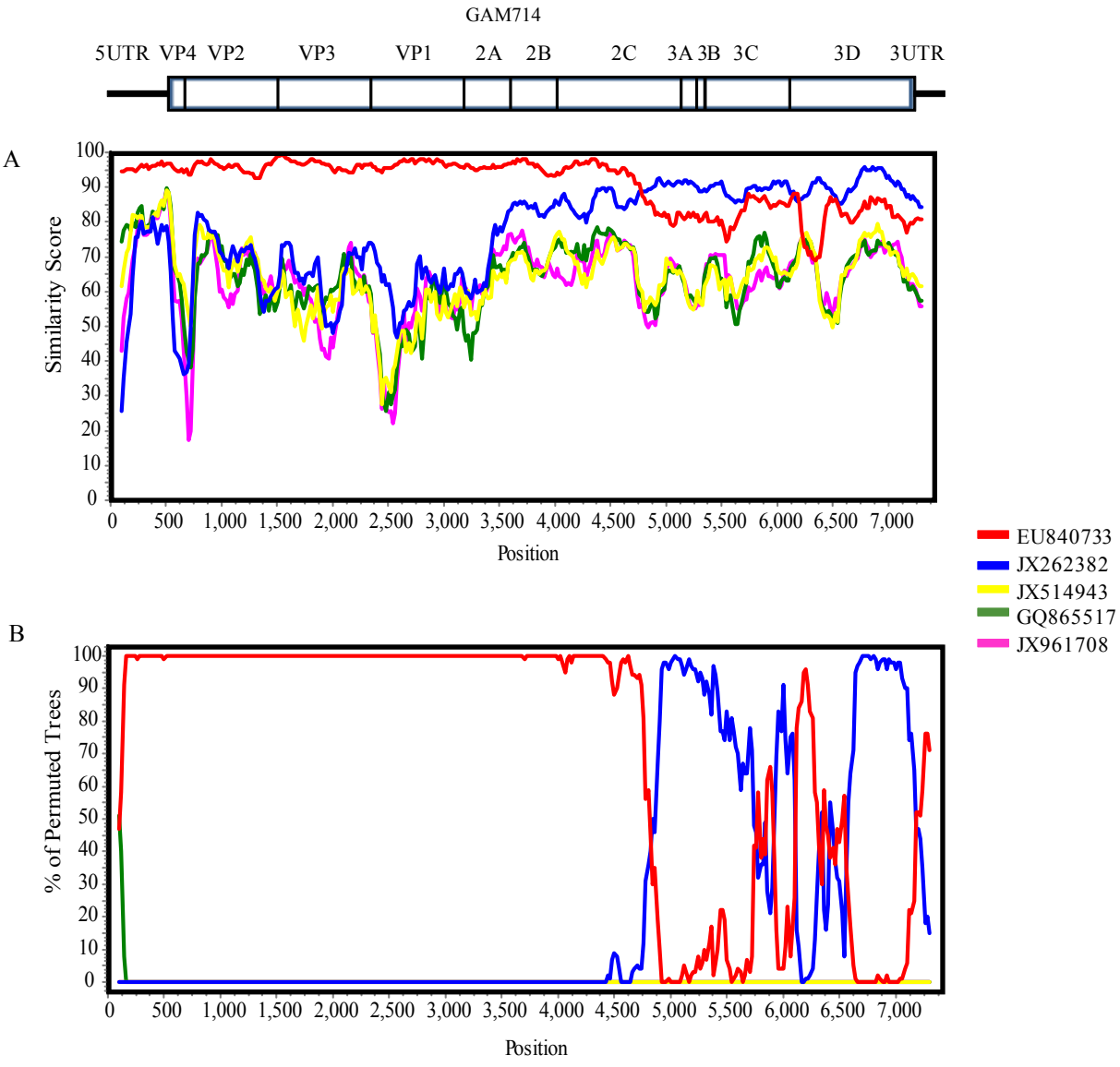
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Similarity Score
Position
B
% of Permuted Trees
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JX262382
JX514943
GQ865517
JX961708

Supplemental Table 1. VP1 sequences of clade A and clade B viruses in GeneBank as of January 2013.

Accession/serotype/strain	Source
KC344834 EV-C113 BBD83	**STOOL**
KC344833 EV-C113 BBD48	**STOOL**
JX538169 CV-A19 BAN/2008/14731	STOOL
JX538147 CV-19 BAN/2008/717820	STOOL
JX538146 CV-A19 BAN/2008/717819	STOOL
CV-A19 BBD48	**STOOL**
CV-A19 BBD66	**STOOL**
JX538106 CV-A22 BAN/2007/14690	STOOL
JX538106 CV-A22 BAN/2007/14690	STOOL
JX538032 CV-A22 BAN/2008/717914	STOOL
JX514943_EV-C105	STOOL
JX514942_EV-C116	STOOL
JX219564 EV-113 BOL/02-10660A	STOOL
JX219563 EV-113 BOL/02-11681A	STOOL
JX219543 EV-113 BOL/02-10821	STOOL
JX219542 EV-113 BOL/02-10649A	STOOL
JX219541 EV-113 BOL/02-10824	STOOL
JX219540 EV-113 BOL/02-10816A	STOOL
JX174177_CV-A1_KS-ZPH01F/XJ/CHN/2011	STOOL
JX174176_CV-A1_HT-THLH02F/XJ/CHN/2011	STOOL
JX139840 CV-A22 A22-F-2332/N.Nov./RU/2010	STOOL
JX139839 CV-A22 CA22-F-2307/N.Nov./RU/2010	STOOL
JX139838 CV-A22 CA22-F-1825/N.Nov./RU/2010	STOOL
JX139837 CV-A22 CA22-F-1799/N.Nov./RU/2008	STOOL
JX139836 CV-A22 CA22-F-14864/N.Nov./RU/2007	STOOL
JX139835 CV-A1 isolate CA1-F-14953/N.Nov./RU/2009	STOOL
JX139818 CV-A22 CA22-F-1463/N.Nov. Dzerzhinsk/RU/2011	STOOL
JX139817 CA-A22 F-2416/N.Nov. Liskovo/RU/2010	STOOL
JX139816 CA22-F-2205/N.Nov./RU/2010	STOOL
JN588566 HEV-C116 F-2390/N.Nov/RU/2009	STOOL
JN588566 EV-C116 F-2390/N.Nov/RU/2009	STOOL
JN588565. HEV-C116 F-1597/N.Nov/RU/2008	STOOL
JN542510_CV-A22_438913	STOOL
DQ995648_CV-A22 USA75-10624	STOOL
DQ995647_CV-A22_ban99-10427	STOOL
DQ455609 CV-A1 strain Germany/922/2005	STOOL
CV-A22 BDD58	**STOOL**
CV-A22 BBD01	**STOOL**
CV-A1 BDD34	**STOOL**
AY919412 CV-A22 10427 VP1	STOOL
AF499643_CV-A22_Chulman	STOOL
AF499641_CV-A19_8663_	STOOL
AF499635 CV-A1_Tompkins	STOOL
AB686524_EV-C104_AK11 2011	NS*
JX982259 EV-C104 Pavia262-11228	NS

JX982258 EV-C104 Pavia68-10804B	NS
JX982257 EV-C104 Pavia 261-9570	NS
JX982256 EV-C104 Pavia 264-16291	NS
JX982255 EV-C104 Pavia263-11230	NS
JX982254 EV-C104 Pavia260-9210	NS
JX982253 EV-C104 Pavia 259-7712	NS
JX961709_EV-C118_ISR38	NS
JX961708_EV-C118_ISR10	NS
GU131227 EV-C109 4733	NS
GU131226 EV-C109 1578	NS
GU131225 EV-C109 4323	NS
GU131224 EV-C109 2751	NS
GQ865517_EV-C109_NICA08-4327 2008	NS
EV-C104 GAM738	**NS**
EV-C104 GAM735	**NS**
EV-C104 GAM724	**NS**
EV-C104 GAM714	**NS**
EV-C104 GAM693	**NS**
EU840733_EV-C104_CL-12310945_	NS
JQ317293_EV-C105 ROM31	NS
JQ317292_ EV-C105 BU77	NS
JQ317291_ EV-C105 BU5	NS
JN900470_EV-C109 L87/HUN/2007	NS
JX393302_EV-C105 PER153	NS
JX393301_EV-C118 PER161	NS
JX262382_EV-C117 LIT22 2011	NS
JX173883 EV-C104 MI1768	NS
JX173882 EV-C104 MI1276	NS
JX173881 EV-C104 PD444	NS
JX173880 EV-C104 MI560	NS
JX173879 EV-C104 MI46	NS

Samples generated in this study are shown in bold
*NS – nasal or nasopharyngeal sample

Supplemental Table 2. Consensus primers used to generate genome sequence of each serotype.

SEROTYPE	DIRECTION, COORDINATES*	FWD PRIMER 5'-3'	REV PRIMER 5'-3'
CV-A1	FWD5, REV1000	GGTTGTTCCCACCCCAGAG	AGWGGGCCACTCACCATA
	FWD 970, REV1880	GGAGCCTGTGGRTATAGTGATAG	GCCTGGTACTGCATTCATTG
	FWD 1800, REV2500	GGAACACCAGARATACACATACCA	TGTCAATAGCGGCCTCAA
	FWD 2400, REV3300	GCTTTGTYAGYGCRTGYAATGA	ATTGCCATGTRTARTCRTCCCA
	FWD 2900, REV4450	CCCCACTGCGTAGTTATGTG	CTGGTTCAATGCGGTGTTT
	FWD 4390, REV4590	GATGGTTRGCRATAAARGCAAG	TGCTCTTCCAATRAGRCCYGT
	FWD 4450, REV5200	AAGACCAAACACCGCATTGA	ATCACYTTRCCRGTDGCCAT
	FWD5062, REV5892	CCAGGGTCCAATTGAATTTAA	CCGTTACCGCCTACGTGTA
	FWD 5800, REV6705	GGGGAGGTAGRCCCACMAA	TTCAADGCTTCRAACCAWGC
	FWD 5864, REV7200	GTTCAGCAGTWGGRTGTGA	CCTCCGAATTAAAAGAAAAT
CV-A19	FWD5, REV1000	GGTTGTTCCCACCCCAGAG	AGWGGGCCACTCACCATA
	FWD1, REV845	TTAAAACAGCTCTGGGGTTGT	TTGCTTTGCTGCTGAATTACT
	FWD650, REV1150	ACCRASTACTTTGGGTGTCCGTG	CCGGYAAYTTCCASCACCA
	FWD1074, REV1800	TCCGGTTGATGCTCCAACT	GGTAAAGCACATGGCGATT
	FWD1700, REV2300	GCACCAGTRACACARGGGTTA	GGCTCCWGGTGGGACAAC
	FWD1750, REV3015	AACCATGGCCACTCCTGG	CGCATTTCCTGTGGTGTAGAAT
	FWD2195, REV3000	CCCACCTAATATGGGACATTG	GATACCAACAAAGGGATGGA
	FWD2900, REV3690	AGGAGCCCCWATCCCWCA	GAGGATTCCACCACARTCACC
	FWD3307, REV4450	AACCCSCATGTAATGACAGC	CTGGTTCAATGCGGTGTTT
	FWD4390, REV4590	GATGGTTRGCRATAAARGCAAG	TGCTCTTCCAATRAGRCCYGT

	FWD4450, REV5200	AAGACCAAACACCGCATTGA	CTACCCAGCCCTGTTGTTT
	FWD4450, REV5877	AAGACCAAACACCGCATTGA	ATCACCTTWCCAGTTGCCAT
	FWD5500, REV6475	GTCTATGACAGGGTTGCTGTACTC	TGCYTTGTTCYACTTTTGATT
	FWD6336, REV6970	CCCAAGAAAAAGAGAGACATCCTC	GTGTGGGTAAGATGCAATAACA
	FWD5864, REV7200	GTTCAGCAGTWGGRTGTGA	CCTCCGAATTAAAAGAAAAT
CV-A22	FWD5, REV1000	GGTTGTTCCCACCCCAGAG	AGWGGGCCACTCACCATA
	FWD860 REV1600	CAAGATCCMTCCAAATTTACTGA	GAACTACYAAGCCCCAATTRTT
	FWD1400 REV2500	TGGGGTTATGGCTGGTAAC	TCGGCCACTTTTGGCATT
	FWD2400, REV3300	GCTTTGTYAGYGCRTGYAATGA	ATTGCCATGTRTARTCRTCCCA
	FWD2900, REV4450	CCCCACTGCGTAGTTATGTG	CTGGTTCAATGCGGTGTTT
	FWD4390, REV4590	GATGGTTRGCRATAAARGCAAG	TGCTCTTCCAATRAGRCCYGT
	FWD4400, REV5200	AAGACCAAACACCGCATTGA	ATCACYTTRCCRGTDGCCAT
	FWD5062, REV5892	CCAGGGTCCAATTGAATTTAA	CCGTTACCGCCTACGTGTA
	FWD5864, REV7200	GTTCAGCAGTWGGRTGTGA	CCTCCGAATTAAAAGAAAAT
EV-C113	FWD5, REV1000	GGTTGTTCCCACCCCAGAG	AGWGGGCCACTCACCATA
	FWD650, REV1150	ACCRASTACTTTGGGTGTCCGTG	CCGGYAAYTTCCASCACCA
	FWD1000, REV1700	AATGGCCGCGTTTCATCA	GGTAACCCCTGCGCACTG
	FWD1550, REV2454	CCATAGCACCWATGTGYTGTG	CCAGCCTCCTGTCTAATAAAAG
	FWD2400, REV3350	GCTTTGTYAGYGCRTGYAATGA	ATTGCCATGTRTARTCRTCCCA
	FWD2958 REV3883	CAAGCATGGGATGATTACACA	CTCTGARATTTTRTTWGCCACTTC
	FWD3669 REV4400	CCAATCCTGGAGACTGTGGT	TGCGRTGTTTGGTCTTGAA
	FWD4334, REV5303	TTTTCAACAACATCAGATGGCTA	GCCACCACTGCGAAAGTG
	FWD5203, REV5600	GGCGGGAATGGTCTATGTG	GCCTCATTCCTATCTAGGGTCAC
	FWD5500, REV6475	GTCTATGACAGGGTTGCTGTACTC	TGCYTTGTTCYACTTTTGATT

	FWD6336, REV6970	CCCAAGAAAAAGAGAGACATCCTC	GTGTGGGTAAGATGCAATAACA
	FWD6587, REV7415	GGTTCAGCAGTWGGRTGTGA	CCCCTCCGAATTAAAAGAAAAT
EV-C104	FWD1, REV720	TTAAAACAGCYTGRGGGTTGTTC	TTCGTGGGTTCCATTGTTC
	FWD650, REV1150	ACCRASTACTTTGGGTGTCCGTG	CCGGYAAYTTCCASCACCA
	FWD1057, REV2157	GCTCAATTCCCATGAGGCAA	CGCACCAGGTGGGCAGTA
	FWD2200, REV3000	GCAACAGGAAAGATCCTGCT	GTTGGCGTTTCCGGTGGT
	FWD2800, REV 3739	CCGCAARCTAGAAATGTTCAC	CACTCCCTCACCACCTGCT
	FWD3665, REV4470	CCGGTGACTGTGGGGGTATA	GGGAGTATGTGCTTGTGTTGG
	FWD4450, REV5304	ACCAAACACCGCATTGAACC	CGCGCATCCCTGAGACTACTAC
	FWD5173, REV6232	GGCAGAGAGTACTGTAAAGGTCA	TGCTCAGTGGGGATATCCAA
	FWD5860, REV7410	TATGGCCACMGGAAAAGTGA	CCTCCGAATTAAAAGAAAATTTACC
	FWD5173, REV6232	GGCAGAGAGTACTGTAAAGGTCA	TGCTCAGTGGGGATATCCAA
	FWD5860, REV 7420	TATGGCCACMGGAAAAGTGA	GGCCTCCGAATTAAAAGAAAAT
	FWD6238, REV7420	TGCCTGGAGGATGCCATGTA	GGCCTCCGAATTAAAAGAAAAT

*Represents approximate coordinates in the genome

All primers were used in PCR with the following conditions:

1) 95^{O}C for 15 minutes 2) 10 cycles of 95^{O}C for 30 seconds, 60^{O}C for 30 seconds (with 1^{O}C decrease at each cycle) and 72^{O}C for 2 minutes 3) 35 cycles of 95^{O}C for 30 seconds, 50^{O}C for 30 seconds and 72^{O}C for 2 minutes.