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AN AVIAN CORONAVIRUS ASSOCIATED WITH FULMINATING DISEASE OF GUINEA FOWL

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Summary

Fulminating disease of Guinea fowl is an acute enteritis characterized by a very high mortality. This disease has been reported for decades in French guinea fowl industry and although its viral origin was previously suspected, the virus remained unknown. Here, we reproduced experimentally the disease by inoculating guinea poults with intestinal content of diseased birds and identified the causative agent using unbiased high throughput sequencing (1).

Introduction

X disease is an acute enteritis has been reported for decades in French guinea fowl industry. This disease also called fulminating disease is characterized by a very high mortality and generally pancreatic degeneration. First investigations have been carried out in the 1980s' to identify the etiological cause of the disease. While a few candidates have been proposed, none had been clearly detected in affected birds (2,3,4). In this study, we reproduced experimentally the disease by inoculating guinea poults with intestinal content of diseased birds and identified a gammacoronavirus as most likely the causative agent (1).

Material and Methods

We investigated field cases of guinea fowl fulminating disease in 2010 and 2011 and collected intestinal content from diseased birds.We inoculated6 weeks old guinea poults with clarified and filtered (0.45 µm mesh) intestinal content. Three groups of five birds were constituted: (i) the first group was orally inoculated with the field filtered sample, (ii) the second group was placed in the same isolator as inoculated birds, (iii) the third group was housed in a distinct isolator and

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was not infected: it was our negative control group. Viral particles of intestinal contents were concentrated by ultracentrifugation and nuclease treatment(RNase (Ambion, 20µg/mL) and DNase (Invitrogen, 10U/µL)). RNA and DNA were extracted and a (RT-)PCR was optimized to generate PCR products of 300bpthanks to tagged random primers previously described (5). An unbiased metagenomic analysis was performed by using MiSeq plateform (Illumina, San Diego, CA, USA). Data reads were analysed using GAAS software (http://gaas.sourceforge.net/) with an E-value of 10^{-3}. Phylogenic analysis was performed using MEGA4.

Results

In our *in vivo* experiment, all inoculated and contact birds (groups 1 and 2)showed clinical signs (severe prostration, decrease in water and feed consumptions)and died or had tobe sacrificed within 6 days (Table 1). The unbiased high-throughput sequencing analysis identified an avian coronavirus which was confirmed by coronavirus-specific RT-PCR on samples from both experimentally infected birds and spontaneous field cases.476,430 sequences were generated, of which 10.8 % matched known viral sequences; and 7.5 % of the eukaryotic viral reads were similar to avian coronaviruses sequences.Blast and phylogenetic analyses on the virus genome showed that this fulminating enteritis virus is indeed a coronavirus (named guinea fowl coronavirus, GfCoV) that clusters with gammacoronaviruses and especially Turkey coronavirus (TCoV). GfCoVspike(S) gene was interestingly more similar to North American TCoV than to European (French) TCoV strains S genes.

Discussion

We have identified a gammacoronavirus in French guinea fowls that we named gfCoV. The very high mortality associated with the fulminating disease in the field may suggest a poor adaptation of the pathogen to its host, which greatly contrasts with previously characterized enteritic avian gammacoronaviruses such as TCoV so far associated with limited morbidity. The epidemiological reservoir of gfCoV still needs to be clarified and further analyses are warranted to fully assess its phylogenetic relationship with other gammacoronaviruses.

In conclusion we showed that an avian coronavirus is the agent of “fulminating disease” of guinea fowl. Coronaviruses cause enteritis in different avian species. Both pathological and epidemiological patterns of this peracute infection are still to be clarified.

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Table 1: Clinical signs after experimental infection of guinea fowls with gfCoV. Green: absence of clinical signs; yellow: severe prostration, decrease in water and feed consumptions; red: death. BF: bursa of Fabricius.

Batch	Experimental follow-up									
Inoculated birds (group 1)		**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**Clinical and necropsic recording**
	A1									Degenerative pancreas +++ / atrophy Thymus & BF
	A2									-
	A3									-
	A4									Degenerative pancreas +++ / atrophy Thymus & BF
	A5									Degenerative pancreas +++ / atrophy Thymus & BF
Contact birds (group 2)	C1									-
	C2									Mucoïd enteritis
	C3									Degenerative pancreas +++ / atrophy Thymus & BF
	C4									Degenerative pancreas +
	C5									-
Control birds (group 3)	T1									Lameness
	T2									-
	T3									-
	T4									-
	T5									-

Full genome sequence of French guinea fowl coronavirus associated with fulminating disease

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Abstract

Guinea fowl coronavirus (gfCoV), a recently characterized avian coronavirus, was identified from outbreaks of fulminating disease (peracute enteritis) in guinea fowls in France. The full-length genomic sequence was determined to better understand its genetic relationship with avian coronaviruses. The full-length coding genome sequence was 26,985 nucleotides long with 11 open reading frames and no hemagglutinin-esterase gene: a genome organization identical to that of turkey Coronavirus [5′ UTR – replicase (ORFs 1a, 1ab) – spike (S) protein – ORF3 (ORFs 3a, 3b) – small envelop (E or 3c) protein – membrane (M) protein – ORF5 (ORFs 4b, 4c, 5a, 5b) – nucleocapsid (N) protein (ORFs N and 6b) − 3′ UTR]. This is the first complete genome sequence of a gfCoV and confirms that the new virus belongs to group III coronaviruses.

Coronaviruses (CoVs) are enveloped viruses with positive-sense, non-segmented RNA genomes of 27 to 32 kb. CoVs infect a wide range of hosts causing various degrees of morbidity and mortality. Group I Coronaviruses (Alphacoronaviruses) contain viruses that infect for example humans (HCoV-229E and HCoV-NL63) but also cats and dogs (with feline CoV and canine CoV, respectively), or pigs (with the porcine transmissible gastroenteritis virus, TGEV for example). Similarly, group II CoVs (Betacoronaviruses) may infect humans (examples: HCoV-OC43, HCoV-HKU1, Severe Acute Respiratory Syndrome (SARS), or Middle East Respiratory Syndrome (MERS) virus), horses (with ECoV), or cattle (with BCoV). In contrast, group III CoVs (Gammacoronaviruses) primarily infect birds: chickens, peafowl, and partridges harbour infectious bronchitis virus (IBV) while turkeys have turkey CoV (TCoV) and guinea fowl may be infected with guinea fowl CoV (gfCoV). Gammacoronavirus strains have however been isolated from a whale and a wild felid (Gavier-

widen D, 2012). Group IV CoVs (Deltacoronaviruses) have been detected in birds (with BuCoV, MuCoV, SpCoV, etc), or pigs (with PorCoV) (Chan et al., 2012). Interestingly CoVs of the Groups I, II, and IV have been detected in Chiroptera (bats), thought to be the reservoir of CoVs (Balboni et al., 2012; Chen et al., 2011).

In the present study we focused on a new member of the group III CoVs, gfCoV, and aimed at sequencing its full genome to better understand its molecular relationship with gammacoronaviruses.

To determine the full genome of GfCoV/FR/2011, we first analysed the data generated on a MiSeq Illumina platform and previously described (Liais et al., 2014). Briefly, pooled intestinal contents of experimentally infected guinea fowl poults were clarified, ultracentrifuged, and treated with nucleases to concentrate viral material. RNA was extracted, and a random RT-PCR was performed to generate unbiased PCR products of about 300 bp (Liais et al., 2014; Victoria et al., 2009). The sequences generated that matched with avian coronaviruses sequences, as determined using GAAS software (Angly et al., 2009), were extracted for further analysis and visualized using Integrative Genomics Viewer (IGV) with the closest Blast hit as reference genome: TCoV MG10 (accession number: EU095850) (Thorvaldsdottir et al., 2013). Primers were designed based on the known sequence data to amplify missing genome fragments by PCR. Sanger sequencing was then performed with PCR primers. The full genome sequence was submitted to EMBL and was attributed the following accession number: (in process). Sequence analysis was carried out using BioEdit version 7.0.8.0 (Hall, 1999), Muscle for the alignment (Edgar, 2004), and Mega version 5.05 for the phylogeny (Tamura et al., 2011).

The sequences were assembled into one contiguous coding sequence of 26,985 nucleotides was obtained. The entire genome had a GC content of 38.3%, identical to the turkey coronavirus (TCoV) MG10 genome (Gomaa et al., 2008).

GfCoV and TCoV genomes have the same organization: (i) a 5' untranslated region (UTR), (ii) two large slightly overlapping ORFs coding for the replicase: 1a and 1ab, (iii) gene coding for the spike (S) protein, (iv) ORF3 (ORFs 3a, 3b), (v) gene coding for the small envelop (E or 3c) protein, (vi) gene coding for the membrane (M) protein, (vii) ORF5 (ORFs X (4b and 4c), 5a, 5b), (viii) genes coding for the nucleocapsid (N) protein (ORFs N and 6b), and (ix) 3′ UTR (Table 1). While some viral proteins were of the exact same size such as 3a, 3b, 4b, 5a, and N with 57, 64, 94, 65, and 409 aa, respectively; variability was observed for the other proteins but not more between gfCoV and the TCoV available genomes than among TCoV genomes. Interestingly, gfCoV/FR/2011 harboured a shorter small envelop protein than its TCoV counterparts (Table 1). Further studies are warranted to understand the impact of avian coronaviruses protein sizes in the biology of the viruses.

Phylogenetic analysis on the full genome of GfCoV/FR/2011 showed it clearly clustered with North American TCoV strains (Figure1, supported by a high bootstrap value of 500), as was observed when a phylogeny of the S gene only had been carried out (Liais et al., 2014). The genetic distance between gfCoV/FR/2011 and TCoV ranged between 10.7% and 11.4%, while these distances were larger between gfCoV/FR/2011 and the representative IBV strains tested as expected: 13.5% to 15.0% (Table 2). A simplot analysis comparing the GfCoV/FR/2011 full genome to its closest TCoV and infectious bronchitis virus (IBV) Blast hits showed that the 3 genomes are highly similar throughout the genome (74 to 100% similarity, with no significantly higher identity of GFCoV/FR/2011 with TCoV or IBV

genomes), except for the S gene (Figure2). GfCoV S gene was indeed more closely related to its closest TCoV than IBV strain but also more similar to both viruses on the S gene than on the rest of its genome (Figure 2), suggesting a recombination event as was hypothesized for the origin of TCoV (Jackwood et al., 2010).

The present study showed that gfCoV/FR/2011 harbours a genome organization very similar to that of TCoV strains. In addition, and again like TCoV, gfCoV/FR/2011 likely originated from a recombination event between an IBV-like (or TCoV-like) virus that would have given most of its genome and a so far unknown CoV that would have contributed by its spike gene. Despite the similarity of their genomes and their enteric tropism, TCoVs cause little clinical signs while gfCoVs are usually associated with extremely high mortalities. Further studies are ongoing to understand the host range of gfCoV/FR/2011 and its determinants of pathogenicity.

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Table 1. Genes and coding regions for gfCoV/FR/2011

ORF	Location (gfCoV)	Size in nt (gfCoV)	Size in aa (gfCoV)	Size in aa (TCoV/VA-74/03)[1]	Size in aa (TCoV/TX-GL/01)[1]	Size in aa (TCoV/TX-1038/98)[1]	Size in aa (TCoV/IN-517/94)[1]	Size in aa (TCoV/ ATCC)[2]	Size in aa (TCoV/ 540)[2]	Size in aa (TCoV/ MG10)[3]
5' UTR*	1-463	>463	-	-	-	-	-	-	-	-
1a	464-12,307	11,844	3,948	3,947	3,949	3,950	3,952	3,957	3,945	3,951
1ab (/*1b*)	464-12,280 and *12,280-20,346*	19,884 *8,067*	6,628 *2,689*	6,596	6,602	6,602	6,605	*2,654*	*2,652*	6,601
S	20,294-23,914	3,621	1,207	1,226	1,225	1,224	1,226	1,203	1,203	1,226
3a	23,917-24,087	171	57	57	57	57	57	57	57	57
3b	24,090-24,281	192	64	64	64	64	64	64	64	64
E (3c)	24,265-24,540	276	92	99	109	99	99	103	99	99
M	24,543-25,214	672	224	223	225	223	223	223	222	223
4b	25,218-25,499	282	94	94	94	94	94	94	94	94
4c	25,423-25,533	111	37					52	56	

5a	25,578-25,772	195	65	65	65	65	65	65	65	65
5b	25,772-26,011	240	80	82	82	92	82	82	80	82
N	25,957-27,183	1,227	409	409	409	409	409	409	409	409
6b	27,191-27,445	255	85					74	73	
3’ UTR*	27,447	27,471	-	-				-	-	-

* Incomplete sequences, nt: nucleotides, aa: amino acids

[1] as described in (Jackwood et al., 2010)

[2] as described in (Cao et al., 2008) with 1b described rather than 1ab (size in aa in italic font)

[3] as described in (Gomaa et al., 2008)

Table 2. Estimates of evolutionary distances (in number of substitutions per site) over sequence pairs between IBV, TCoV, and GfCoV/FR/2011.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*
1-GF-CoV/FR/2011		*0.002*	*0.002*	*0.003*	*0.002*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.002*	*0.003*	*0.003*	*0.003*
2-EU022526_TCoV-ATCC	**0.111**		*0.002*	*0.001*	*0.002*	*0.002*	*0.002*	*0.001*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*	*0.002*
3-GQ427176_TCoV/TX-1038/98	**0.108**	0.073		*0.001*	*0.002*	*0.001*	*0.002*	*0.001*	*0.002*	*0.002*	*0.003*	*0.003*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*	*0.002*
4-GQ427174_TCoV/TX-GL/01	**0.114**	0.079	0.039		*0.001*	*0.001*	*0.001*	*0.001*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.001*	*0.001*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.002*
5-GQ427173_TCoV/VA-74/03	**0.112**	0.076	0.046	0.046		*0.001*	*0.002*	*0.001*	*0.002*	*0.003*	*0.002*	*0.002*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.002*
6-EU095850_MG10	**0.111**	0.075	0.050	0.045	0.031		*0.001*	*0.001*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*	*0.002*
7-GQ427175_TCoV/IN-517/94	**0.107**	0.069	0.056	0.057	0.050	0.043		*0.001*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*	*0.002*
8-EU022525_TCoV-540	**0.114**	0.076	0.069	0.070	0.066	0.062	0.039		*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*	*0.002*
9-FN430415_NGA/A116E7/2006	**0.135**	0.138	0.128	0.134	0.133	0.134	0.131	0.138		*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*
10-FN430414_ITA/90254/2005	**0.136**	0.146	0.138	0.145	0.143	0.145	0.140	0.147	0.100		*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*
11-KF377577_strain_4/91_vaccine	**0.147**	0.150	0.140	0.145	0.146	0.149	0.144	0.150	0.107	0.113		*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.003*	*0.003*	*0.002*	*0.002*	*0.002*
12-AJ311317_Beaudette_CK	**0.149**	0.134	0.134	0.132	0.137	0.137	0.133	0.139	0.109	0.113	0.111		*0.001*	*0.001*	*0.001*	*0.001*	*0.002*	*0.002*	*0.001*	*0.002*	*0.002*	*0.002*	*0.003*	*0.002*
13-AY851295_strain_Mass_41	**0.150**	0.136	0.131	0.128	0.138	0.136	0.133	0.140	0.108	0.114	0.113	0.067		*0.001*	*0.001*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*
14-AY641576_isolate_Peafowl/GD/KQ6/2003	**0.150**	0.137	0.133	0.128	0.137	0.135	0.134	0.138	0.107	0.115	0.106	0.068	0.020		*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*
15-GU393336_serotype_Holte	**0.139**	0.120	0.124	0.129	0.129	0.126	0.122	0.131	0.100	0.109	0.109	0.085	0.085	0.087		*0.001*	*0.001*	*0.002*	*0.001*	*0.001*	*0.002*	*0.002*	*0.002*	*0.002*
16-GU393337_serotype_Iowa_97	**0.139**	0.119	0.121	0.125	0.128	0.125	0.121	0.129	0.100	0.108	0.105	0.086	0.086	0.088	0.006		*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.002*	*0.002*	*0.002*
17-GU393334_serotype_Gray	**0.136**	0.112	0.113	0.118	0.114	0.113	0.107	0.115	0.095	0.104	0.106	0.087	0.083	0.085	0.071	0.071		*0.000*	*0.001*	*0.002*	*0.001*	*0.002*	*0.002*	*0.002*
18-GU393338_serotype_JMK	**0.136**	0.112	0.113	0.119	0.114	0.113	0.107	0.116	0.095	0.104	0.105	0.087	0.082	0.085	0.071	0.071	0.003		*0.001*	*0.002*	*0.001*	*0.002*	*0.002*	*0.002*
19-KF696629_strain_Connecticut_vaccine	**0.137**	0.109	0.099	0.106	0.106	0.107	0.101	0.117	0.093	0.104	0.103	0.075	0.075	0.076	0.068	0.069	0.064	0.063		*0.001*	*0.001*	*0.002*	*0.002*	*0.001*
20-AY514485_serotype_California_99	**0.148**	0.129	0.101	0.108	0.112	0.119	0.115	0.129	0.102	0.112	0.105	0.102	0.100	0.100	0.089	0.084	0.081	0.079	0.055		*0.001*	*0.002*	*0.002*	*0.001*
21-GQ504721_strain_Arkansas_Vaccine	**0.145**	0.123	0.095	0.104	0.107	0.119	0.113	0.126	0.098	0.110	0.089	0.095	0.095	0.089	0.083	0.077	0.065	0.064	0.057	0.048		*0.001*	*0.001*	*0.001*
22-FJ888351_strain_H120	**0.149**	0.140	0.117	0.120	0.127	0.133	0.134	0.142	0.097	0.112	0.088	0.085	0.084	0.068	0.090	0.086	0.091	0.087	0.081	0.077	0.058		*0.001*	*0.002*
23-GU393332_serotype_Delaware_072	**0.150**	0.139	0.117	0.123	0.126	0.137	0.137	0.142	0.117	0.131	0.107	0.126	0.128	0.113	0.119	0.112	0.115	0.114	0.108	0.099	0.077	0.056		*0.001*
24-GQ504723_strain_Georgia_1998_Vaccine	**0.149**	0.130	0.101	0.104	0.112	0.125	0.120	0.131	0.117	0.129	0.119	0.120	0.118	0.117	0.111	0.105	0.101	0.101	0.081	0.077	0.058	0.088	0.044	

The number of base substitutions per site from averaging over all sequence pairs are shown. Standard error estimate(s) are shown above the diagonal, in italic font. Estimates of evolutionary distances over sequence pairs between gfCoV/FR/2011 and its counterparts are indicated in

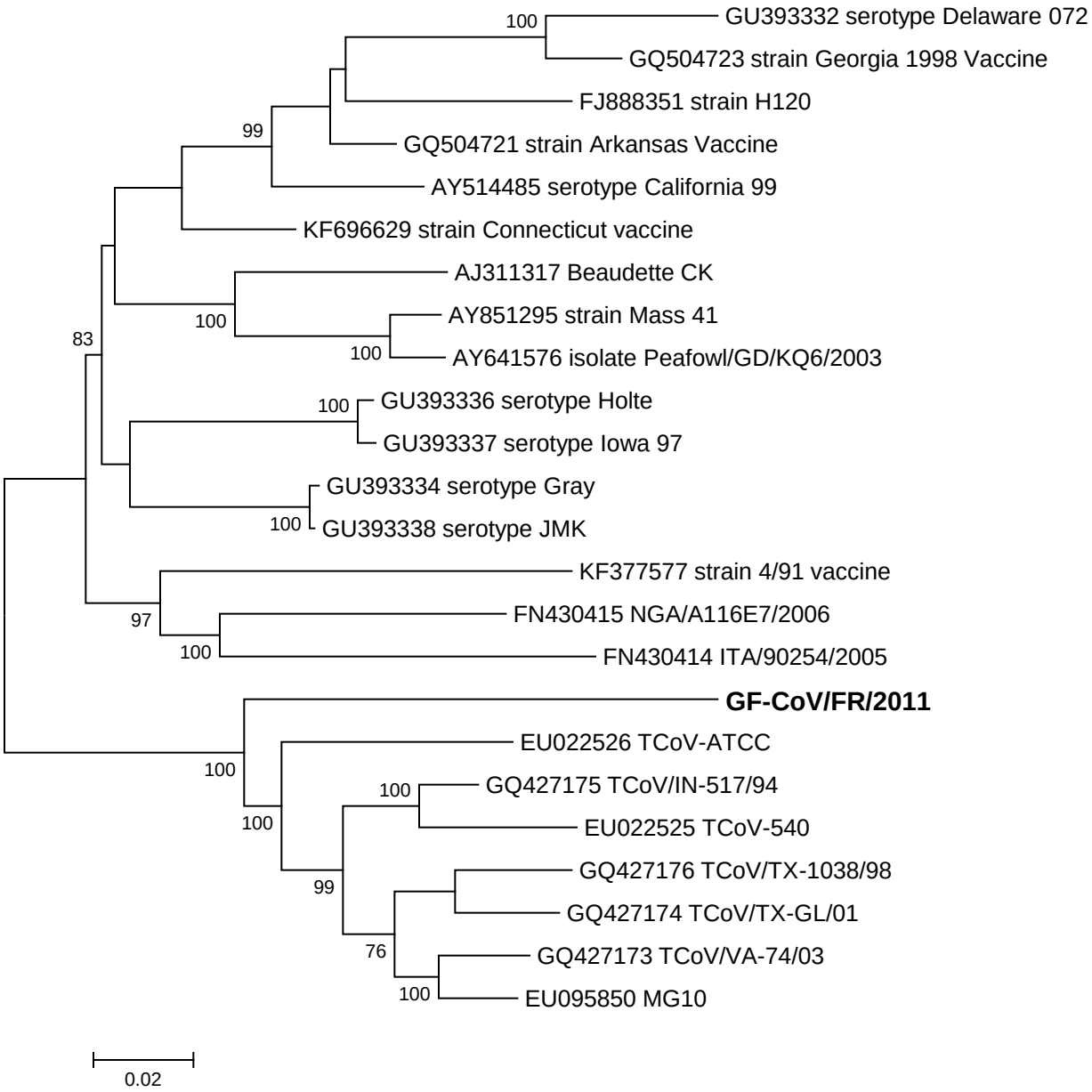
bold font. The Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated.

Figure legends

Figure 1. Phylogenetic analysis of the complete genomes of GFCoV/FR/2011 (in bold font) in relation to all available full genomes of turkey coronaviruses (TCoV) and full genomes of representative infectious bronchitis viruses (IBV) at the nucleotide level. The tree was generated using MEGA 5.05 and the Maximum Likelihood method. Bootstrap values (500 replicates) >75 are indicated on the nodes.

Figure 2. Simplot analysis of full genomic sequence for GFCoV/FR/2011 (query) and its closest TCoV (in blue) and IBV (in red) blast hits. The spike gene area is indicated on the plot.

Figure 1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GQ504723 strain Georgia 1998 Vaccine
FJ888351 strain H120
GQ504721 strain Arkansas Vaccine
AY514485 serotype California 99
KF696629 strain Connecticut vaccine
AJ311317 Beaudette CK
AY851295 strain Mass 41
AY641576 isolate Peafowl/GD/KQ6/2003
GU393336 serotype Holte
GU393337 serotype Iowa 97
GU393334 serotype Gray
GU393338 serotype JMK
KF377577 strain 4/91 vaccine
FN430415 NGA/A116E7/2006
FN430414 ITA/90254/2005
GF-CoV/FR/2011
EU022526 TCoV-ATCC
GQ427175 TCoV/IN-517/94
EU022525 TCoV-540
GQ427176 TCoV/TX-1038/98
GQ427174 TCoV/TX-GL/01
GQ427173 TCoV/VA-74/03
EU095850 MG10
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83
100
100
100
100
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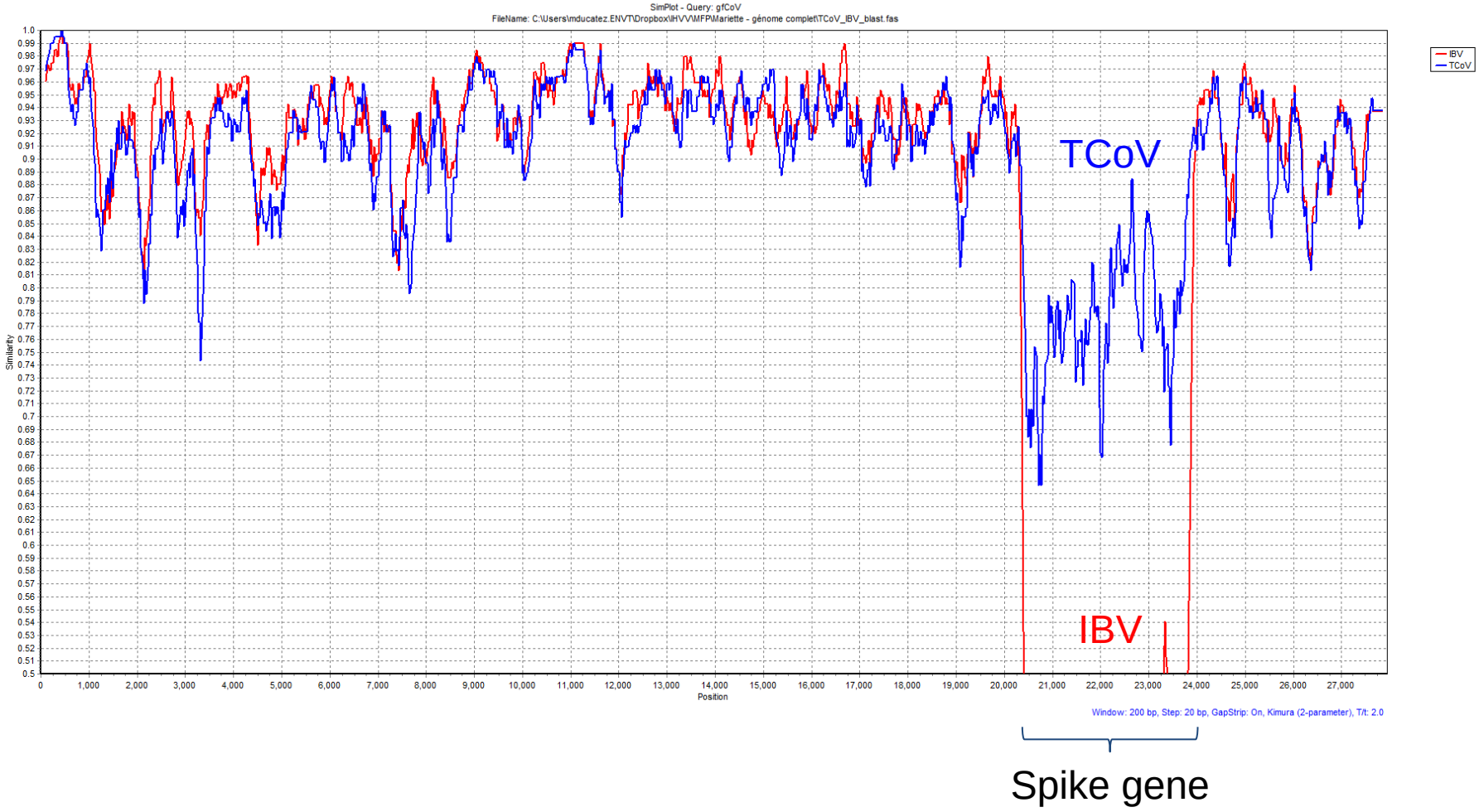
100
100
100
100
99
76
100
0.02

Figure 2


SimPlot - Query: gfCoV
FileName: C:\Users\mducatez.ENVT\Dropbox\IHVV\MFP\Mariette - génome complet\TCoV_IBV_blast.fas
IBV
TCoV
Similarity
Position
TCoV
IBV
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2.0
Spike gene