First Detection and Frequent Occurrence of Equine Hepacivirus in Horses on the African Continent

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Abstract

Since the discovery of equine hepacivirus (EqHV) in 2011, the virus has been detected in horse populations from more than twelve countries across five continents. EqHV seroprevalence has been reported to be as high as 61.8% and EqHV ribonucleic acid (RNA) prevalence to range between 0.9% and 34.1%. Molecular and serological indications of EqHV infection have never been reported in equids on the African continent. Therefore, investigation of EqHV prevalence in South African horses and subsequent viral genetic characterization contribute to a better understanding of the global epidemiology of this virus.

In a cross-sectional study, serum samples from 454 Thoroughbred foals (aged 58-183 days) were analyzed for anti-EqHV non-structural protein 3 (NS3)-specific antibodies (abs) with a luciferase immunoprecipitation system (LIPS) and for EqHV RNA by quantitative real-time polymerase chain reaction (qRT-PCR). Farms of origin (n=26) were situated in South Africa's Western Cape Province. The associations between EqHV infection state and farm of origin, foal gender and foal age were subsequently described. Furthermore, nested PCRs were performed on parts of the 5'UTR, NS3 and NS5B genes of 17 samples. Samples were sequenced and phylogenetic analyses were conducted.

The population's seroprevalence was 83.70% and RNA was detected in 7.93% of samples. Increasing foal age was associated with decreasing ab prevalence and increasing prevalence of

EqHV RNA. Sequences from South African EqHV strains did not show in-depth clustering with published sequences of EqHV isolates from particular continents.

In conclusion, EqHV is present in the South African Thoroughbred population and appears more prevalent than reported in other horse populations worldwide.

Keywords

Non-primate hepacivirus

Hepacivirus A

Equid

Phylogenetic analysis

South Africa

Thoroughbred

1. Introduction

Equine hepacivirus (EqHV) is one of 14 species belonging to the genus *Hepacivirus* in the family *Flaviviridae* (Smith et al., 2016). This virus, which has also been referred to as canine hepacivirus, non-primate hepacivirus and most recently, Hepacivirus A, represents the closest related homologue of hepatitis C virus (HCV) (Pfaender et al., 2014; Smith et al., 2016). Hepaciviruses have been isolated from a variety of mammalian and non-mammalian host species, including dogs (Kapoor et al., 2012), horses (Burbelo et al., 2012; Pfaender et al., 2014), rodents, bats (Drexler et al., 2013), monkeys (Lauck et al., 2013),

cattle (Baechlein et al., 2015; Corman et al., 2015), donkeys (Walter et al., 2017) and a catshark (Shi et al., 2016).

Vaccine development against HCV is hampered because the virus has a narrow species tropism and is genetically divergent, with seven recognized genotypes. Compared to other hepaciviruses, EqHV not only has a higher sequence similarity to HCV, but also displays liver tropism and can induce chronic infections (Pfaender et al., 2015; Scheel et al., 2015). In contrast to HCV, circulating EqHV strains have been described as closely related (Pronost et al., 2017) and an established immune response against primary viral challenge protects the host against reinfection (Pfaender et al., 2017).

Evidence of EqHV has been found in horse populations and commercial horse sera on five continents: Asia, Australia, Europe, North and South America (Burbelo et al., 2012; Drexler et al., 2013; Elia et al., 2017; Gemaque et al., 2014; Lyons et al., 2012; Matsuu et al., 2015; Pfaender et al., 2015; Postel et al., 2016; Pronost et al., 2017; Reuter et al., 2014; Tanaka et al., 2014). The seroprevalence has been reported to be as high as 61.8% (Reichert et al., 2017) and the RNA prevalence to range between 0.9% (Lyons et al., 2014) and 34.1% (Tanaka et al., 2014). However, no information is available about the occurrence and the genetic diversity of EqHV in African horses.

In this study the occurrence of EqHV was investigated for the first time in South African Thoroughbred foals. Serum samples from 454 foals were analysed for the presence of anti-EqHV NS3-specific abs and EqHV RNA and revealed the highest seroprevalence described in a horse population to date. Phylogenetic analyses revealed a close relation to other EqHV isolates circulating worldwide.

2. Materials and Methods

2.1 Study design and population

In this cross-sectional study, serum samples from Thoroughbred foals (aged 58-183 days) were analysed. The foals originated from 26 farms in the Western Cape Province of South Africa. Samples were collected during January and February 2017, as part of the South African Thoroughbred Breeders Association's (TBA's) annual Thoroughbred foal identification project. Of all the foals identified by the TBA in 2017, 454 were included in this study. Samples were selected based on availability of serum and compliance with regulations for export of horse serum from South Africa to Europe. Thoroughbred horses in the Western Cape Province were considered to be representative of the South African Thoroughbred population.

Considering an estimated target population of 3400 Thoroughbred foals born in South Africa annually, the sample size was calculated. The expected prevalence of foals positive for RNA was set to 12-16%, and the expected prevalence of foals positive for abs was set to 50%, given a confidence interval of 95%. As the prevalence of RNA-positive foals was estimated with a precision of +/- 3%, 398-491 foals had to be tested, whereas estimating the prevalence of ab-positive foals with a precision of +/- 5% required a sample size of 346 foals. Hence, the investigation of 454 foals was considered to be sufficient to estimate the EqHV prevalence.

Frozen samples were shipped to Germany and stored at -80°C prior to analyses for the presence of anti-EqHV NS3-specific abs and EqHV RNA. The study was approved by the Animal Ethics Committee of the University of Pretoria (Study V021-17).

2.2 Detection of anti-EqHV NS3-specific abs

Samples were analysed in duplicate for the presence of anti-EqHV NS3-specific abs, using the LIPS assay as described previously (Burbelo et al., 2009). Relative light units (RLU) were measured with a plate luminometer (LB 960 XS3; Berthold, Freiburg, Germany). The threshold value, above which samples were regarded as ab-positive, was calculated for each plate by using the mean value plus three standard deviations (SD) of the wells containing only buffer A, Renilla luciferase extract and protein A/G beads.

2.3 Detection of EqHV RNA

Viral RNA was extracted using the High Pure Viral RNA Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Extracted RNA was stored at -80°C until further analysis or was directly transcribed into complementary DNA (cDNA) by applying the PrimeScriptRTMaster Mix Kit (TaKaRa, Kusatsu, Japan). All cDNA samples were stored at −20°C before being used for qRT-PCR. For the SYBR Green based qRT-PCR, the SYBR®Premix Ex Taq™ II kit (TaKaRa, Kusatsu, Japan) was used, in combination with the previously described primers targeting the 5′ untranslated region (5′UTR) (Burbelo et al., 2012). A standard curve for the quantification of RNA copies was assessed by serial dilution of a plasmid containing the EqHV 5′UTR based on the EqHV isolate NPHV-NZP-1 (JQ434001.1). Measurement of fluorescence was conducted with a LightCycler 480 (Roche, Mannheim, Germany).

2.4 Data analysis

Descriptive analyses were performed to study associations between EqHV infection state and farm of origin, foal gender and foal age.

2.5 Sequencing and phylogenetic analyses

Seventeen samples, which tested positive for EqHV RNA, were used for sequence analyses. Therefore, nested PCRs targeting parts of the 5'UTR, NS3 and NS5B domains were conducted as previously described (Lyons et al., 2012; Elia et al., 2017) and sent to GATC Biotech for Sanger sequencing. Previously published sequences were retrieved from the GenBank database by searching for (((((((NPHV) OR equine hepacivirus) OR non primate hepacivirus) OR nonprimate hepacivirus) OR non-primate hepacivirus) AND ((UTR) OR complete)) NOT pegivirus) NOT cloning vector and NS3 or NS5B, respectively. All sequences were aligned with MUSCLE and primer sequences were deleted. Incomplete sequences from GenBank were excluded from further analyses. Phylogenetic analyses were conducted by using the Maximum-Likelihood method by MEGA7 (Kumar et al., 2016) based on the General Time Reversible model (Nei and Kumar, 2000). Gamma distributed with Invariant sites (G+I) was set for rates among sites with a number of six discrete gamma categories. All sequences were uploaded to the NCBI database with accession numbers (MG892467 - MG892483, MH632169 - MH632202).

3. Results

3.1 Detection of anti-EqHV NS3-specific abs and EqHV RNA in African equine sera

In this study, serum samples from South African Thoroughbred foals (Fig. 1A) were analysed for anti-EqHV NS3-specific abs by LIPS assay and for EqHV RNA by qRT-PCR. Samples were collected from foals on 26 farms of origin. All farms were located in the Western Cape Province of South Africa (Fig. 1B).

The seroprevalence in the study population was 83.70%, with 380 of the 454 samples positive for abs (Fig. 2A). The inner-herd seroprevalence (the average % of seropositive foals per herd) was 85.37%. EqHV RNA was detectable in 7.93% of samples (36/454), with

Fig. 1 Location of sampling. (A) A map of Africa highlighting, in light gray, the location of South Africa. (B) An enlarged map of South Africa highlighting, in light gray, the Western Cape Province. Serum samples were collected from 454 Thoroughbred foals on 26 farms located in the Western Cape Province.

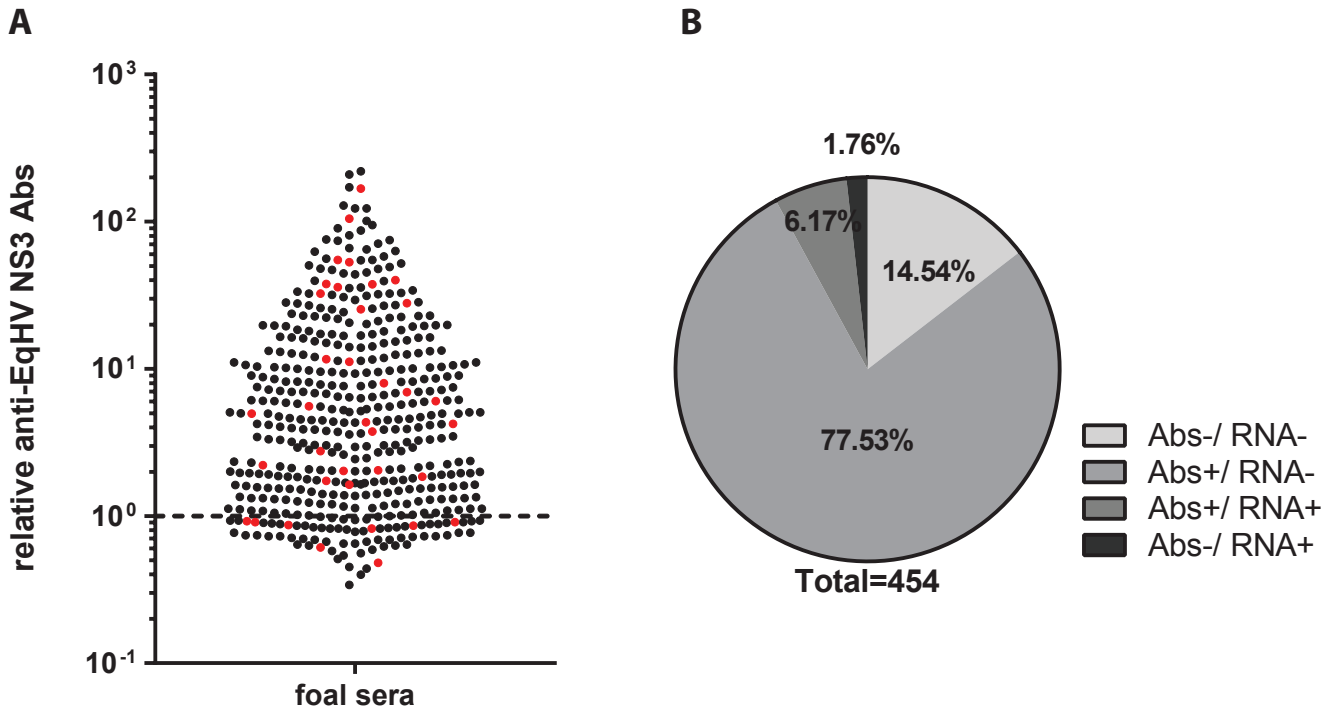

Fig. 2. Prevalence of EqHV infection in 454 Thoroughbred foals. (A) The LIPS ratio for each of the foals. RLU were normalized to fold change over threshold (dotted line). EqHV RNA- positive foals are indicated in red and EqHV RNA-negative foals are indicated in black. (B) Anti-EqHV abs and EqHV RNA results categorized and depicted in a pie chart.

viral loads which ranged from 8.0 x 10^4 to 1.6 x 10^7 RNA copies/ml serum. The inner-herd RNA prevalence (the average % of RNA-positive foals per herd) was 9.26%. The samples were assigned to four categories based on the foals' EqHV infection state: seronegative and RNA negative [Abs-/RNA-]; seropositive and RNA negative [Abs+/RNA-]; seropositive and RNA positive [Abs+/RNA+] and seronegative and RNA positive [Abs-/RNA+]. Three hundred and fifty-two samples (77.53%) contained only abs, but no EqHV RNA. Eight samples (1.76%) contained only RNA, but no abs. Only 66 samples (14.54%) tested negative for both EqHV RNA, as well as abs (Fig. 2B).

3.2 Associations between EqHV infection state and farm of origin, foal gender and foal age

The number of foals per farm varied from 1 to 81 (Table 1). Evidence of EqHV (abs, RNA or both) was detected in samples which originated from 25 of the 26 farms (Table 1). Only in the case of farm I (n=2 foals) did both foals test negative for abs and RNA. For 13 of the 26 farms, evidence of EqHV (abs, RNA or both) was detected in 100% of the foals (Table 1). Herd prevalence (the % of farms with at least one foal in a particular infection-state category) and inner-herd prevalence (the average % of foals in a particular infection-state category per farm) were calculated for the various EqHV infection-state categories, to avoid overestimation of prevalence due to inclusion of farms with low foal numbers (Table 2).

Comparable results were obtained between male and female foals in terms of the different EqHV infection states detected (Fig. 3A). However, more male foals tested positive for RNA (8.97% colts and 6.82% fillies). Similarly, the seroprevalence in colts (84.19%) was higher than in fillies (83.18%).

Table 1 Number of foals and their corresponding EqHV infection state, according to the farm of origin.

Farm		EqHV Infection State							
		Abs-/RNA-		Abs+/RNA-		Abs+/RNA+		Abs-/RNA+	
Name	No. of Foals	n	%	n	%	n	%	n	%
A	31	0	0.00	26	83.87	3	9.68	2	6.45
B	18	5	27.78	13	72.22	0	0.00	0	0.00
C	3	0	0.00	2	66.67	1	33.33	0	0.00
D	27	7	25.93	16	59.26	4	14.81	0	0.00
E	81	10	12.35	65	80.25	4	4.94	2	2.47
F	2	1	50.00	1	50.00	0	0.00	0	0.00
G	23	1	4.35	20	86.96	2	8.70	0	0.00
H	47	9	19.15	32	68.09	4	8.51	2	4.26
I	2	2	100	0	0.00	0	0.00	0	0.00
J	8	3	37.50	3	37.50	2	25.00	0	0.00
K	3	0	0.00	3	100	0	0.00	0	0.00
L	8	0	0.00	8	100	0	0.00	0	0.00
M	6	1	16.67	5	83.33	0	0.00	0	0.00
N	16	1	6.25	15	93.75	0	0.00	0	0.00
O	71	9	12.68	57	80.28	3	4.23	2	2.82
P	2	0	0.00	2	100	0	0.00	0	0.00
Q	10	0	0.00	9	90.00	1	10.00	0	0.00
R	36	9	25.00	25	69.44	2	5.56	0	0.00
S	9	0	0.00	9	100	0	0.00	0	0.00
T	30	8	26.67	22	73.33	0	0.00	0	0.00
U	11	0	0.00	11	100	0	0.00	0	0.00
V	2	0	0.00	1	50.00	1	50.00	0	0.00
W	4	0	0.00	4	100	0	0.00	0	0.00
X	2	0	0.00	1	50.00	1	50.00	0	0.00
Y	1	0	0.00	1	100	0	0.00	0	0.00
Z	1	0	0.00	1	100	0	0.00	0	0.00
Total	**454**	**66**	**14.54**	**352**	**77.53**	**28**	**6.17**	**8**	**1.76**

Table 2 Herd prevalence and inner herd prevalence for the various EqHV infection-state categories

	EqHV Infection State		
	Abs+/RNA-	**Abs+/RNA+**	**Abs-/RNA+**
Herd prevalence	96.15% (25/26 farms)[1]	46.15% (12/26 farms)[2]	15.38% (4/26 farms)[3]
Inner-herd prevalence[4]	76.73%	8.64%	0.62%

[1] = farms with at least one Abs+/RNA- foal; [2] = farms with at least one Abs+/RNA+ foal; [3] = farms with at least one Abs-/RNA+ foal; [4] = average % of foals in this category per herd.

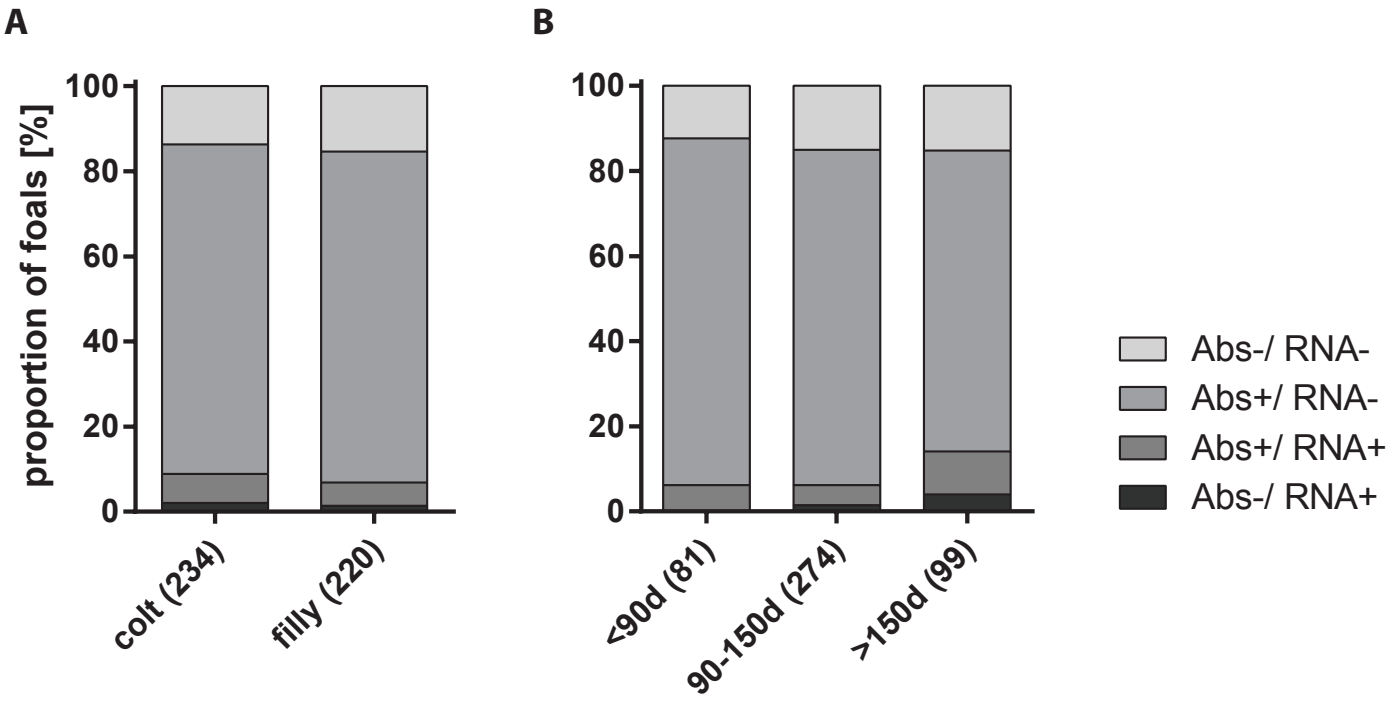

Fig. 3. Associations of EqHV infection state with other factors. (A) Bar diagram with the categories of EqHV infection state displayed according to foal gender and (B) foal age in days (d).

When assigned to three age categories (<90 days; 90-150 days; >150 days; Fig. 3B), more foals older than 150 days tested positive for RNA only (4.04%) than foals younger than 150 days (0% and 1.46%, respectively). Furthermore, fewer foals younger than 90 days were neither positive for abs nor for RNA (12.35%) compared to foals older than 90 days (14.96% and 15.15%, respectively).

3.3 Sequence and phylogenetic analyses

Phylogenetic analyses were conducted with nested PCR and sequencing, using available GenBank sequences which matched the gene sections of the sequences obtained in this study. All amplicons sequenced in this study are available on the NCBI database (Table 3). A maximum likelihood approach was used and a bootstrap was performed with a replicate rate of 1000. Prior to this study, no sequences from samples which originated in Africa had been published. No in-depth clustering of the EqHV 5'UTR (supplementary Fig. 1 and 2), NS3 (Fig. 4 and 5) or NS5B (supplementary Fig. 3 and 4) from South Africa was observable with any sequences from particular continents in this multi-target typing approach. However, the genetic distances between samples from all continents were minor. This argues for the prevalence of only one genotype worldwide, based on these results.

4. Discussion

This study represents the first report of EqHV in horses on the African continent. Furthermore, the EqHV seroprevalence of 83.70% is the highest reported to date. Published data reported EqHV seroprevalence to be as high as 61.8% (Elia et al., 2017; Lu et al., 2016; Pronost et al., 2017; Reichert et al., 2017). The 7.93% prevalence of EqHV RNA detected, is within the range of 0.9% to 34.1% reported previously (Elia et al., 2017; Lu et al., 2016; Pronost et al., 2017; Reichert et al., 2017). To the authors' knowledge, inner-herd

Table 3 Specifications of samples used for phylogenetic analyses

No.	Farm	Age [days]	Sex	Antibody Status	Viral Load [RNA copies/ ml serum]	Accession Numbers 5'UTR / NS3 / NS5B
1	A	136	M	+	$7.4x10^5$	MH632169 / MG892467 / MH632186
2	A	80	F	+	$1.2x10^6$	MH632170 / MG892468 / MH632187
3	H	94	F	+	$1.1x10^6$	MH632171 / MG892469 / MH632188
4	H	108	F	+	$1.6x10^7$	MH632172 / MG892470 / MH632189
5	H	109	M	-	$5.5x10^6$	MH632173 / MG892471 / MH632190
6	X	160	F	+	$2.5x10^6$	MH632174 / MG892472 / MH632191
7	D	132	M	+	$1.1x10^6$	MH632175 / MG892473 / MH632192
8	G	86	F	+	$1.5x10^6$	MH632176 / MG892474 / MH632193
9	O	84	M	+	$1.7x10^6$	MH632177 / MG892475 / MH632194
10	O	86	M	+	$2.6x10^6$	MH632178 / MG892476 / MH632195
11	J	151	M	+	$1.6x10^6$	MH632179 / MG892477 / MH632196
12	R	182	M	+	$3.1x10^6$	MH632180 / MG892478 / MH632197
13	E	173	F	-	$6.2x10^6$	MH632181 / MG892479 / MH632198
14	E	96	F	+	$2.2x10^6$	MH632182 / MG892480 / MH632199
15	Q	75	M	+	$1.9x10^6$	MH632183 / MG892481 / MH632200
16	V	118	F	+	$1.2x10^6$	MH632184 / MG892482 / MH632201
17	R	156	F	+	$1.3x10^6$	MH632185 / MG892483 / MH632202

F = female, M = male

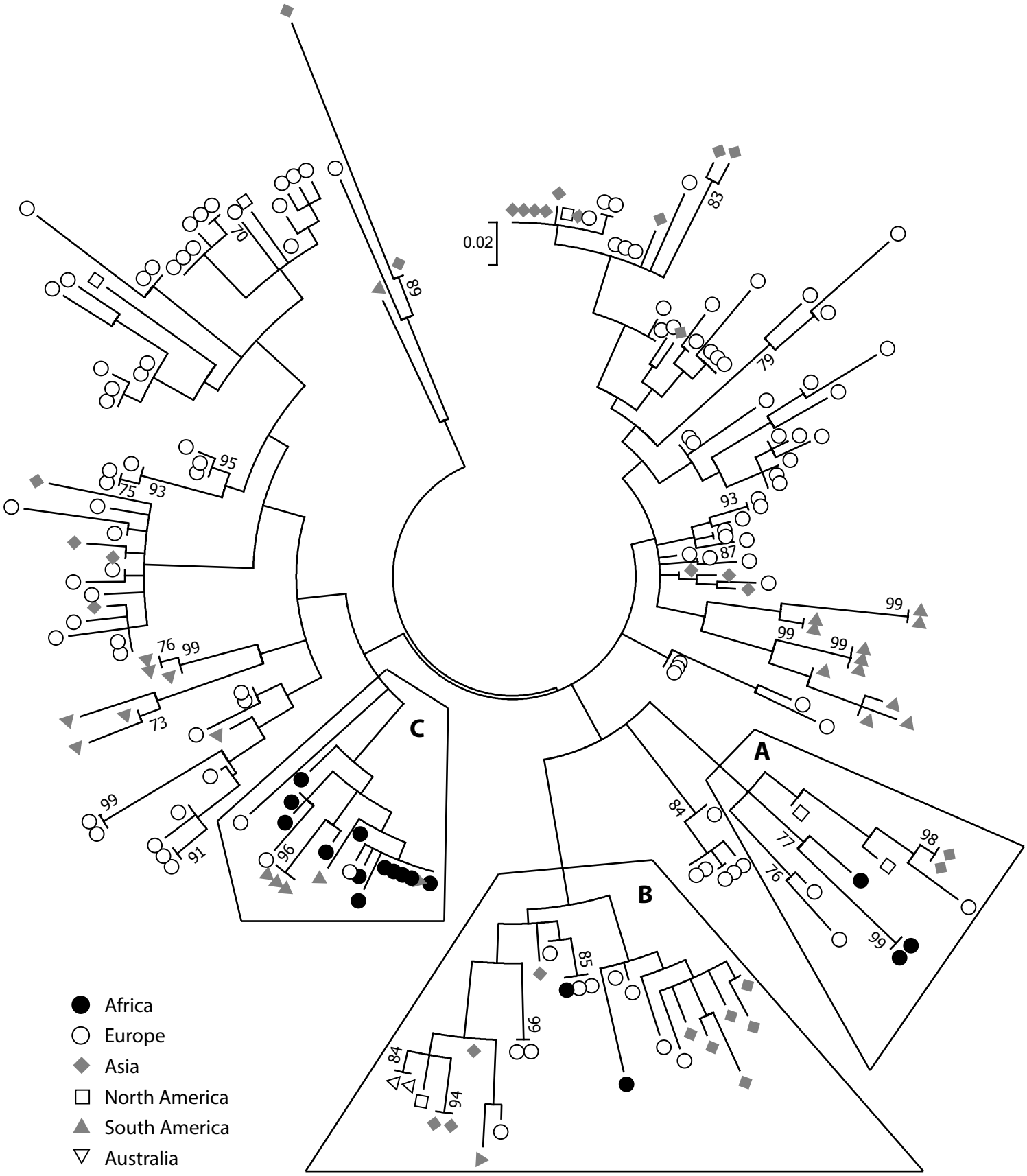

Fig. 4. The maximum-likelihood phylogeny is based on partial NS3 sequences of EqHV. In addition to sequences obtained from South African foals, the phylogenetic tree contains sequences for EqHV retrieved from GenBank, as described in the Materials and Methods section. The analysis involved 198 nucleotide sequences. All positions containing gaps and missing data were eliminated, whereby a total of 122 positions were included in the final dataset. Bootstrap values <70% are not shown. The symbol legend indicates symbols and the corresponding continent which a sequence originated from. The scale bar illustrates the number of substitutions per site. Framed sections, including sequences obtained in this study, are shown in detail in Fig. 5.

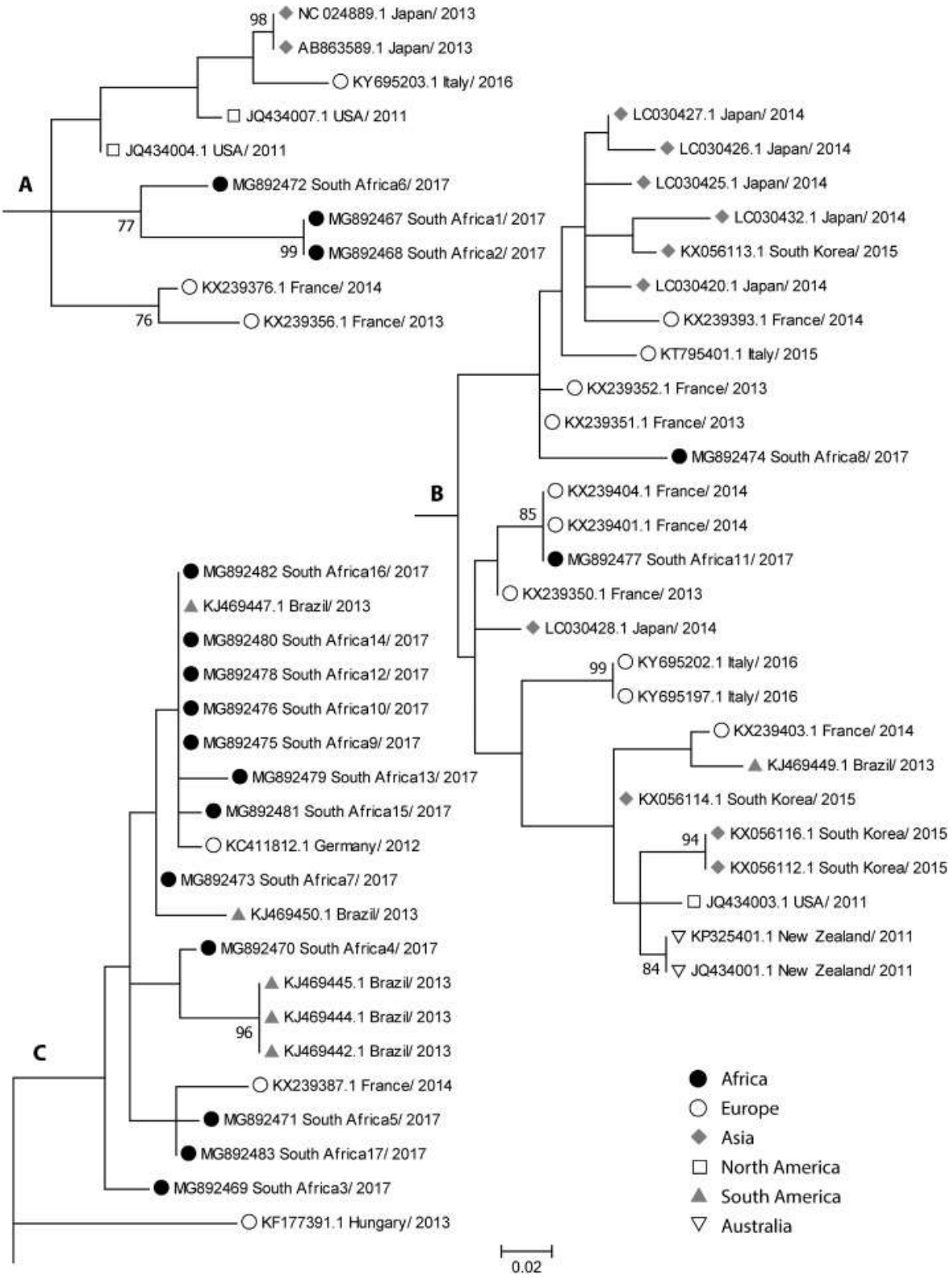

Fig. 5. Detailed views of the complete phylogenetic tree based on partial-NS3 sequences of EqHV. (A-C) Each panel illustrates a part of the complete tree, including the accession number, country and year of each sequence. Additionally, sequences acquired in the present study are labelled with the number of the foal which the isolate originated from (refer to Table 3). The symbol legend indicates symbols and the corresponding continent which a sequence originated from. The scale bar illustrates the number of substitutions per site.

seroprevalence and inner-herd RNA prevalence have not been reported previously for EqHV.

The study population consisted of young Thoroughbred horses, aged between 58 and 183 days. The occurrence of EqHV infection has been found to be significantly higher in Thoroughbred populations compared to other breeds (Pfaender et al., 2015; Pronost et al., 2017; Reichert et al., 2017). Despite the lack of data on EqHV occurrence in other horse populations in Africa, this study suggests that EqHV prevalence in South African Thoroughbreds is higher than that reported in horse populations on other continents.

The data related to foal age and EqHV infection state support the occurrence of acute and chronic EqHV infections in young horses (Gather et al., 2016a). A decrease in ab prevalence and an increase in acute infections [Abs-/RNA+] observed in older foals, could be related to the transfer of maternal abs to foals via colostrum (Gather et al., 2016b). Anti-EqHV maternal ab titres in foals wane between three and six months of age (Gather et al., 2016b; Gather et al., 2016a). In addition to maternal abs, early EqHV infection followed by seroconversion and viral clearance cannot be excluded as another cause of the frequently observed seropositive and RNA-negative [Abs+/RNA-] infection state. Clearance of acute EqHV infection within less than two months, frequently with simultaneous emergence of abs, was reported in at least 60% of an infected horse cohort (Pfaender et al., 2015). The co-presence of viraemia and abs [Abs+/RNA+] is indicative of recent seroconversion or chronic infection. Chronic EqHV infection, despite the presence of abs, has been reported in Thoroughbred foals up to one year of age (Gather et al., 2016a; Gather et al., 2016b). Mechanisms of EqHV transmission remain largely unknown. However, vertical transmission and phylogenetic clustering of EqHV isolates from individuals within their respective herds have been reported - also suggesting a horizontal route of transmission

(Gather et al., 2016b). Here, the EqHV-positive horses did not cluster based on the specific sites, except for number 1 and 2 on farm A (Table 3). The absence of data pertaining to the EHV-status of our study foals and their mares at the time of birth, limited the interpretation of these results. Our data support published data that show age as a risk factor for EqHV infection (Matsuu et al., 2015; Reichert et al., 2017).

Phylogenetic analyses revealed no in-depth clustering with EqHV strains from certain continents, supporting the close relation of circulating EqHV strains worldwide.

5. Conclusion

In conclusion, this study showed that anti-EqHV abs are frequently detected in South African Thoroughbred foals, indicating circulating EqHV with a high prevalence. Furthermore, closely related strains of EqHV are circulating worldwide, suggesting low selection pressure on this virus.

6. Acknowledgements

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8. **Conflict of interest**

None

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