Longitudinal Survey of Astrovirus infection in different bat species in Zimbabwe: Evidence of high genetic Astrovirus diversity

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Abstract

Astroviruses (AstVs) have been discovered in over 80 animal species including diverse bat species and avian species. A study on Astrovirus circulation and diversity in different insectivorous and frugivorous chiropteran species roosting in trees, caves and building basements was carried out at 11 different sites across Zimbabwe. Pooled and individual faecal sampling methods were used for this study, with collection dates ranging from June 2016 to July 2021. In two sites, Magweto and Chirundu, sampling was carried out at monthly intervals from August 2020 to July 2021. Astroviruses and bat mitochondrial genes were amplified using pan-AstVs and CytB /12S RNA PCR systems respectively. Phylogenetic analysis of the *RdRp* Astrovirus sequences revealed a high genetic diversity of astroviruses. All the bat astroviruses tested in this study clustered with the *Mamastrovirus* genus. Two distinct groups of amplified sequences were identified. One group was comprised of sequences isolated from *Hipposideros, Rhinolophus* and *Eidolon helvum* spp. clustered with Human Astrovirus strains: *HuAstV* types1-6, *HuAstV*-MLB1-3 and *HuAstV*-VA-1. The second group comprising the majority of the sequences clustered with different strains of Bat AstVs. Results from the longitudinal study at Magweto and Chirundu showed an overall AstV prevalence of 13.7% and 10.4% respectively. Peaks of AstV infection at Chirundu coincided with the period when juveniles are 4-6 months old. By combining data from our previous work on Coronaviruses, we also anaylzed co-infection of bats with Coronaviruses and Astroviruses at Magweto and Chirundu sites for which the prevalence of co-infection was 2.6% and 3.5% respectively.

53 **Introduction**

54 Astroviruses (AstVs) are non-enveloped single-stranded positive sense RNA viruses with a genome
55 length of approximately 6.2 to 7.7 Kb [1]. They have been described in over 80 non-human species
56 and are classified into two genera: *Mamastrovirus* and *Avastrovirus,* respectively infecting mammals
57 and avian species [2]. According to the International Committee on Taxonomy of Viruses
58 (https://talk.ictvonline.org), *Mamastrovirus* are classified into 19 recognized species, *MAstV*-1 to -19,
59 and two genogroups GI and GII [3]. All classic and novel Human Astrovirus (*HuAstVs*) belong to
60 four different species, *MAstV*-1, *MAstV*-6, *MAstV*-8 and *MAstV*-9 [2,4,5]. Recently, studies have
61 reported AstVs infections in fish and insects [6,7], however bats and wild birds are considered to be
62 natural reservoirs of the *Astroviridae* family [8,9]. Generally *HuAstVs* have been identified as causal
63 agents of acute viral gastrointestinal illness worldwide particularly in children, immunocompromised
64 people and the elderly [6–8]. Of note, in humans, the majority of AstV-associated encephalitis or
65 meningitis illnesses were reported in immunocompromised people [10–12]. Furthermore, beyond this
66 well-known clinical manifestation of AstVs infections, neurovirulent AstVs infections have also been
67 reported in both humans and domestic animals [13,14]. Moreover, studies have reported numerous
68 cross-species transmissions of AstVs [7], where some AstVs detected in humans closely clustered
69 with strains identified in rodents, minks, feline species and ovine [2,5,7]. Bat-borne viruses represent
70 an extensive research field owing to the plethora of viruses hosted by Chiropterans [15]. Furthermore,
71 this mammalian order is known to be persistently infected by astroviruses [16] and this is inclusive
72 of many species of insectivorous bats [17]. To date, a high diversity of bat AstVs from different bat
73 families has been reported worldwide [11,18-19]. Bat AstVs belong to *MAstV*-12, and *MAstV*-14 to -
74 19 species [1]. In Europe a variety of bat species belonging to the Yangochiroptera sub-order have
75 been discovered to harbor several astroviruses [20]. In Africa, bat astroviruses were reported in Egypt,
76 Gabon, Madagascar, Kenya and Mozambique [18, 21–23].

77 AstVs are known to occur in bat species with high prevalence and exceedingly high genetic diversity
78 whereby infections in these hosts are usually non-pathogenic [24,25]. Despite all these studies, large
79 gaps exist on astroviruses prevalence and infection dynamics in bats [9]. Astroviruses prevalence and
80 detection in bat colonies seemed rather correlated to abiotic factors such as seasons and year of
81 sampling and biotic factors such as sex and reproductive status and also viral co-infection [26]. Viral
82 shedding in bats occurs in spatial and temporal pulses that can drive spillover to other animals or
83 humans [27-28]. The varying prevalence of viruses in bats is determined by shedding pulses, where
84 rare or very low prevalences are detected in the absence of shedding pulses and higher prevalence is
85 detected when shedding pulses occur [27,29].

86 Until now, no data was available on genetic diversity and prevalence of circulating AstVs in
87 insectivorous bats in Zimbabwe. In this study we enlarge the spectrum of bat AstVs knowledge in

Africa by identifying and analyzing bat-AstVs, their diversity and prevalence from different bat species colonies according to reproductive season phenology in Zimbabwe. Furthermore, we will also show and highlight the presence of co-infection of bats by astroviruses and coronaviruses (CoVs) and how it varies according to reproductive phenology.

Material and Methods

Sampling approaches and sites

Two different approaches were followed in this study: bat community sampling and individual bat sampling.

Bat community pooled sampling

Between February 2016 and December 2020, faecal samples from both insectivorous and frugivorous bat species were respectively collected in different sites including caves, an ancient mine and trees in Zimbabwe (Figure 1, Table 1).

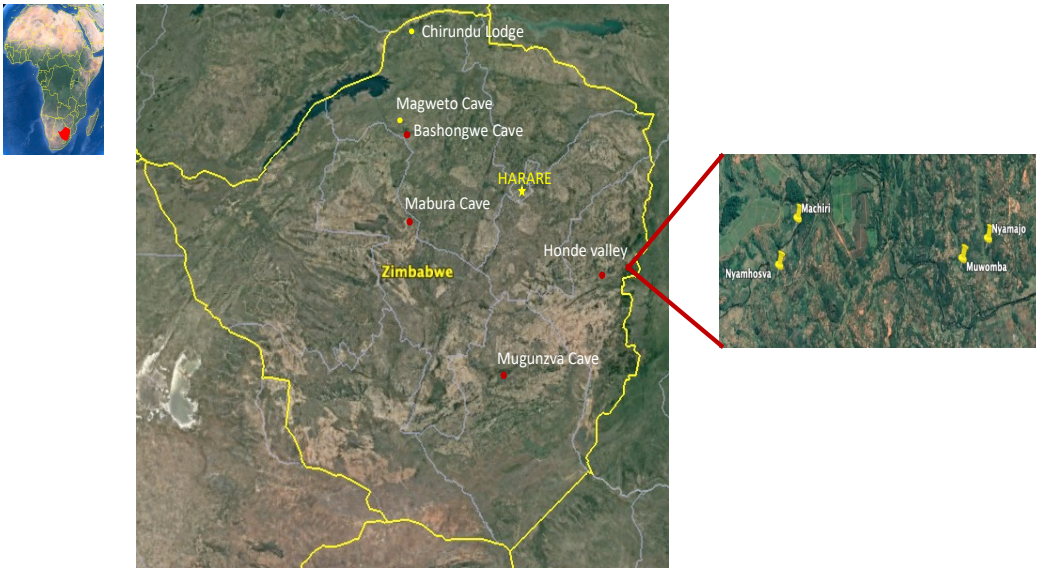


Figure 1: The study sites highlighted on the map show the sampling areas for insectivorous and frugivorous bats colonies across Zimbabwe. In Honde valley, the *Eidolon helvum* colony was established in at least four different sites represented by the rectangle at the right of the figure. Red circles represent community approach study sites; yellow circles represent longitudinal survey sites (individual approach).

108 **Table 1:** Number of Astrovirus positives per site/sampling year in pooled faecal samples from frugivorous and
109 insectivorous bats for the selected 14 sites.

Sampling Sites	Type of roost	Collection Date	Bat Species	Number of collected tubes	Number of Tested pools	Number of positive pools
Magweto Cave	Cave	June 2016	*Hipposideros spp.*	24	8	5
Bashongwe Cave	Cave	March 2018	*Rhinolophus and Hipposideros spp.*	59	7	0
		July 2018		57	20	0
		December 2018		135	45	0
Mabura Cave	Cave	June 2016	*Hipposideros spp.*	36	12	6
		February 2017	*Hipposideros spp.*	22	7	0
Mugunza Cave	Cave	April 2017	*Roussetus aegyptus*	50	13	0
Honde Valley						
Machiri	Trees	August 2017	*Eidolon helvum*	25	9	0
Muwomba		November 2017	*Eidolon helvum*	66	22	0
Nyamajo		November 2017	*Eidolon helvum*	58	19	0
Nyamhosva		November 2017	*Eidolon helvum*	35	11	0
Nyamajo		January 2018	*Eidolon helvum*	17	6	1
Machiri		January 2018	*Eidolon helvum*	7	2	2
Muwomba		January 2018	*Eidolon helvum*	8	2	0
Nyamhosva		January 2018	*Eidolon helvum*	28	10	1
Nyamajo		February 2020	*Eidolon helvum*	92	92	0
Chirundu	Tree	December 2020	*Rhinolophus spp.*	21	21	1
Total				**740**	**306**	**16**

110

111 All these sites were chosen according to the presence of bat colonies and the existence of anthropic
112 activities. For instance, the selected caves and ancient mine are regularly visited by local people to
113 collect bat guano, which is used as fertilizer, and/or to hunt bats for consumption. In Honde valley,
114 frugivorous bats roosting sites in trees were close to maize crops or/and orchards.
115 All sites, except for three, were visited at different times during the sampling period (Table 1). The
116 same sampling method was used at all sites and in every session as previously described by Bourgarel
117 et al, [30]. Briefly, two square meters of plastic sheets were laid down at each site/per sampling
118 session, underneath the bat colonies and left overnight. Six grams of fresh faeces from different bat
119 individuals were collected from each plastic sheet in a 15 ml tube with 6 ml of RNA stabilization
120 solution (*https://protocol-online.org/*). Back in the laboratory, samples were stored at -80°C for
121 subsequent analysis. Of note, for all sampling sessions, personnel protection equipment (double pair
122 of gloves, FFP3 mask, Tyvek overall, goggles, boots) were used in order to respect the biosafety
123 procedures and to protect the sampling teams. Good biosafety practices training are regularly
124 dispensed for our sampling teams.

125 ***Bat individual sampling***

126 Individual bat samples which had already been collected from two study sites for a study on
127 coronaviruses by Chidoti et *al*. were used in this study (Figure 1) [31]. These two sites, one cave
128 (Magweto) and one building basement (Chirundu Farm) had been visited from August 2021 to July
129 2022 on a monthly basis (Figure 1, Table 2) [31]. Unfortunately, this study was conducted during the
130 COVID 19 crisis and we were not able to access the study sites every month as planned owing to the
131 imposed lock downs. Faecal samples had been collected by placing two square meters plastic sheets
132 underneath the bat colonies. Only one faecal dropping (not contaminated by other faeces or urine)
133 per 20 cm^2 was collected, assuming it represented one individual. Faeces were conserved individually
134 in a 1.5 ml tube filled up with 0.5 ml of home-made RNA stabilization solution (*https://protocol-*
135 *online.org/*) and stored at -80°C before further laboratory analyses.

136 **Table 2:** Prevalence of Astroviruses and co-infection with Coronaviruses and confidence intervals (CIs) per
137 month at both Magweto and Chirundu sites in faecal samples from individual sampling of insectivorous bat
138 communities and per reproduction cycle stage.

Site	Reproduction cycle	Month sampled	No of samples tested	No of Astrovirus positives	Prevalence (%) + CI (95%)	No of coinfection CoV and AstV	Prevalence (%) + CI (95%)
Chirundu	**Non-gestation**	August, 2020	153	0	0.0 (0.0-0.2)	0	0.0 (0.0-0.2)
Chirundu	**Pregnancy**	October, 2020	297	3	1.0 (0.3-2.9)	0	0.0 (0.0-1.3)
Chirundu	**Parturition**	November, 2020	241	3	1.2 (0.4-3.6)	1	0.4 (0.05-2.3)
Chirundu	**Lactation**	December, 2020	159	16	10.1 (6.3-15.7)	7	4.4 (2.1-8.8)
Chirundu	**Weaning**	February, 2021	170	29	17.1 (12.1-23.4)	18	10.6 (6.8-16.1)
Chirundu	**4-6 Month juveniles**	March, 2021	240	51	21.3 (16.5-26.8)	19	7.9 (5.1-12.0)
Chirundu	**Non-gestation**	May, 2021	243	47	19.3 (14.8-24.7)	9	3.7 (1.9-6.8)
Chirundu	**Non-gestation**	July,2021	225	30	13.3 (9.5-18.4)	9	4.0 (2.1-7.4)
Overall prevalence			**1728**	**179**	**10.4 (9.0-11.8)**	**63**	**3.6 (3.8-5.8)**
Magweto	**Non-gestation**	September, 2020	348	18	5.2 (3.3-8.0)	1	0.3 (0.05-1.6)
Magweto	**Pregnancy**	October, 2020	257	46	17.9 (13.7-23.0)	7	2.7 (1.3-5.5)
Magweto	**Parturition**	November, 2020	228	31	13.6 (9.7-18.6)	3	1.3 (0.4-3.8)
Magweto	**4-6 Month juveniles**	March, 2021	242	46	19.0 (14.5-24.4)	20	8.3 (5.4-12.4)
Magweto	**Non-gestation**	April, 2021	242	26	10.7 (7.4-15.2)	6	2.5 (1.1-5.3)
Magweto	**Non-gestation**	June, 2021	242	47	19.4 (14.9-24.9)	3	1.2 (0.4-3.6)
Overall prevalence			**1559**	**214**	**13.7 (12.1-15.5)**	**40**	**2.6 (1.8-3.5)**

139

140 Periods of gestation (pregnancy), parturition, lactation, weaning and presence of 4-6 months old
141 juveniles had been determined based on observations (from captures and observation of the roosting
142 bats) combined with literature, as already reported in Chidoti et *al.* [31]. The reproductive season at
143 Chirundu and Magweto site had been observed to begin in September to February for the predominant
144 bat species. The different insectivorous bat families observed at both sites had been found to be
145 synchronous regarding their reproductive cycles, and consensus reproduction periods had been
146 determined based on the literature and observations [31,32].

147 **Nucleic acid extraction and RT-PCR**

148 ***Community samples***.

149 Nucleic acids extraction was done from all faecal samples as previously described [30]. Briefly,
150 biological material (faeces) of three or four sample tubes collected from the same plastic sheet were
151 pooled and transferred in a 50 ml tube with 20 ml of PBS 1X then vigorously mixed. Tubes were
152 centrifuged at 4500 rpm for 10 min. The supernatant was first filtered using gauze in order to eliminate
153 faecal matter and transferred in fresh tubes then re-centrifuged at 4500 rpm for 10 min. The
154 supernatant was filtered through a 0.45 µm filter to remove eukaryotic and bacterial sized particles.
155 Seven millilitres of filtered samples were centrifuged at 250,000 g for 2.5 h at 4°C. The pellets were
156 re-suspended in 600 µl H_20 molecular grade and 150 µl were used to extract RNA and DNA using
157 NucleoSpin® RNA Kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's protocol.

158 ***Individual samples***

159 Nucleic acids were extracted from 200 µl of faecal samples preserved in 0.5 ml RNA stabilization
160 solution using 5X MagMax Pathogen RNA/DNA Kit (ThermoFisher Scientific, Illkirch-
161 Graffenstaden, France), as already described in [30]. The faeces were vortexed vigorously (30Hz)
162 using Retsch MM400 Tissuelyser for 5 min to fully homogenise and mix the faecal particles, followed
163 by centrifugation at 16000 g for 3 min to fully separate the supernatant from the faecal debris. A
164 volume of 130µl of the supernatant was used for the isolation and purification stage of the nucleic
165 acids using Mag Max extraction kit with the automatic KingFisher Duo Prime Purification System
166 extractor (ThermoFisher Scientific, Illkirch-Graffenstaden, France). A final volume of 80µl of eluted
167 RNA/DNA was stored at -80◦C.

168 ***Bat species identification***

169 For all bat samples that tested positive for AstVs, bat species identification was carried out by the
170 amplification and sequencing of mitochondrial C*ytochrome b* gene as previously described by Kocher
171 et *al.* [33]. *S*equences were then compared to available bat sequences in the GenBank database using
172 *Basic Local Alignment Search Tool* (BLAST).

173 ***Astrovirus detection***

174 Reverse Transcription (RT) using random hexamers were done on 5µl of RNA sample template using
175 1µl random hexamers, 0.5µl Oligo dT primer, 0.4µl of dNTPs (10mM) (ThermoFisher Scientific,
176 Illkirch-Graffenstaden, France) and 5.5µl molecular grade water incubated at 65°C for 5 min. This
177 was followed by addition of 4µl of Buffer 5X, 2µl of 0.1M DTT (M-MLV Reverse Transcriptase,
178 Invitrogen, ThermoFisher Scientific) and 1µl of RNAse OUT, incubated at 37°C for 2 min. A volume
179 of 1µl of M-MLV (M-MLV Reverse Transcriptase, Invitrogen, ThermoFisher Scientific, Illkirch-
180 Graffenstaden, France) reverse transcriptase was added to the mixture followed by incubation at 25ºC
181 for 10 min, 37ºC for 50 min and 70ºC for 15 min. The cDNA obtained was then used to partially
182 amplify the *Astrovirus RNA-dependent-RNA polymerase* gene (*RdRp*) by using a semi-nested Pan-

183 Astrovirus PCR system developed by Chu et *al* [16]. Visualization of positive PCR products was
184 performed with agarose gel electrophoresis using SYBR green on a 1% gel. Positive PCR products
185 (422 bp) were gel-agarose or directly purified (Geneclean Turbo Kit, MP Biomedicals, Illkirch-
186 Graffenstaden, France) and then sequenced in both 5' and 3' directions (LGC, Berlin, Germany) by
187 using Sanger method.
188 For the community approach, purified PCR products were cloned by using Topo PCR Cloning kit
189 according to the manufacturer's protocol (ThermoFisher Scientific, Illkirch-Graffenstaden, France).
190 Ten clones per PCR product were sequenced in both 5' and 3' direction using the Sanger method
191 (Eurofins, Germany).
192
193 **Phylogenetic analyses**
194 Bat AstVs overlapping sequences were assembled into contiguous sequences using Geneious
195 software package V. 2021.2.2 (Biomatters Ltd, Auckland, New Zealand). Partial non-concatenated
196 nucleic acid sequences of the new AstVs were aligned using Clustal W [34] implemented in MEGA
197 7 [35], with minor manual adjustments. Ambiguously aligned and divergent regions were excluded
198 from subsequent analyses. Phylogenies were inferred using Maximum Likelihood (ML) method
199 implemented in PhyML V. 3.0 [36]. The suited evolution model was selected by Akaike's Information
200 criterion (AIC) using Topali software [37]. The reliability of branching orders was tested using the
201 bootstrap approach (1000 replicates) [38] and the GTR + F+ I substitution model was determined as
202 the best suited evolution model.
203
204 **Temporal variations of Astrovirus prevalence and bat reproductive phenology**
205 In the same way as in Chidoti et *al*, the prevalence of astrovirus infection was calculated at the
206 community level for small insectivorous bat species from the two longitudinal study sites, Magweto
207 and Chirundu farm [31]. The proportion of RNA AstV-positive samples were estimated per month
208 and site with 95% confidence intervals (CI) using Wilson score test
209 *(https://epitools.ausvet.com.au/ciproportion)* [39].
210 The influence of the different phases of the bat reproductive cycle stages (periods of pregnancy, par-
211 turition/lactation, weaning, weaned juveniles of 4 to 6 months old) and of *Coronavirus* infection/shed-
212 ding on the prevalence of astroviruses was tested by running a generalized linear mixed model
213 (GLMM) for each site (Magweto and Chirundu) as described in Chidoti et *al* [31]. Parturition and
214 lactation were analysed as one season because the observations did not allow the separation of the
215 two periods as they were interlinked. The response variable with a binomial distribution was the
216 AstVs PCR result of the samples, and the explanatory variables with fixed effects were the different
217 phases of the reproduction cycle. Given Coronavirus PCR results on the same samples were available

218 through our previous study (see below), Coronavirus status was also integrated into the model as
219 fixed variable to investigate any effect of co-infection on AstVs infection/shedding. The reproductive
220 phases were coded as 1 if it was during the corresponding reproduction phase and 0 if it was not. A
221 session identification code was included as a random effect to control for repeated measures from the
222 same sampling session to account for clustered samples collected.

223 ***Astrovirus and Coronavirus co-infection***

224 Coronavirus data, produced by Chidoti et *al* [31], on the same sample sets were used in this study to
225 assess the AstVs and CoVs co-infection in bat communities from Magweto and Chirundu sites and a
226 descriptive analysis of prevalences was carried out (**Table 2**).

227

228 **Results**

229 ***Astrovirus detection in pooled sampling sites***

230 The sampling was carried out from June 2016 to December 2020 (Table 1) at the selected sites. At
231 Magweto cave site, 24 samples were collected. In Bashongwe collection was carried out in March,
232 July and December 2018, and 251 samples were collected. For Mabura cave site, a total of 58 samples
233 were collected, in 2016 and 2017, with sampling only carried out once in both years. In Mugunza
234 cave site, a once off sampling session was done in 2017 and a total of 50 samples were collected. In
235 the Honde Valley site, 336 samples were collected under nine *Eidolon helvum* roost trees (Table 1).
236 In Magweto, five of the eight pooled samples (62.5%) tested positive for AstV. In Bashongwe cave,
237 all the 72 samples collected tested negative for AstV (Table 1). In Mabura, Six (31.6%) out of 19
238 pooled samples tested positive for AstVs and all of the 13 samples from Mugunza tested for AstVs
239 were negative (Table 1). In Honde Valley, 173 pooled samples were tested for AstVs and only four
240 (2.3%) were positive (Table 1). In Chirundu Baobab tree only one (4.8%) out of the 21 samples
241 collected was positive for AstV.

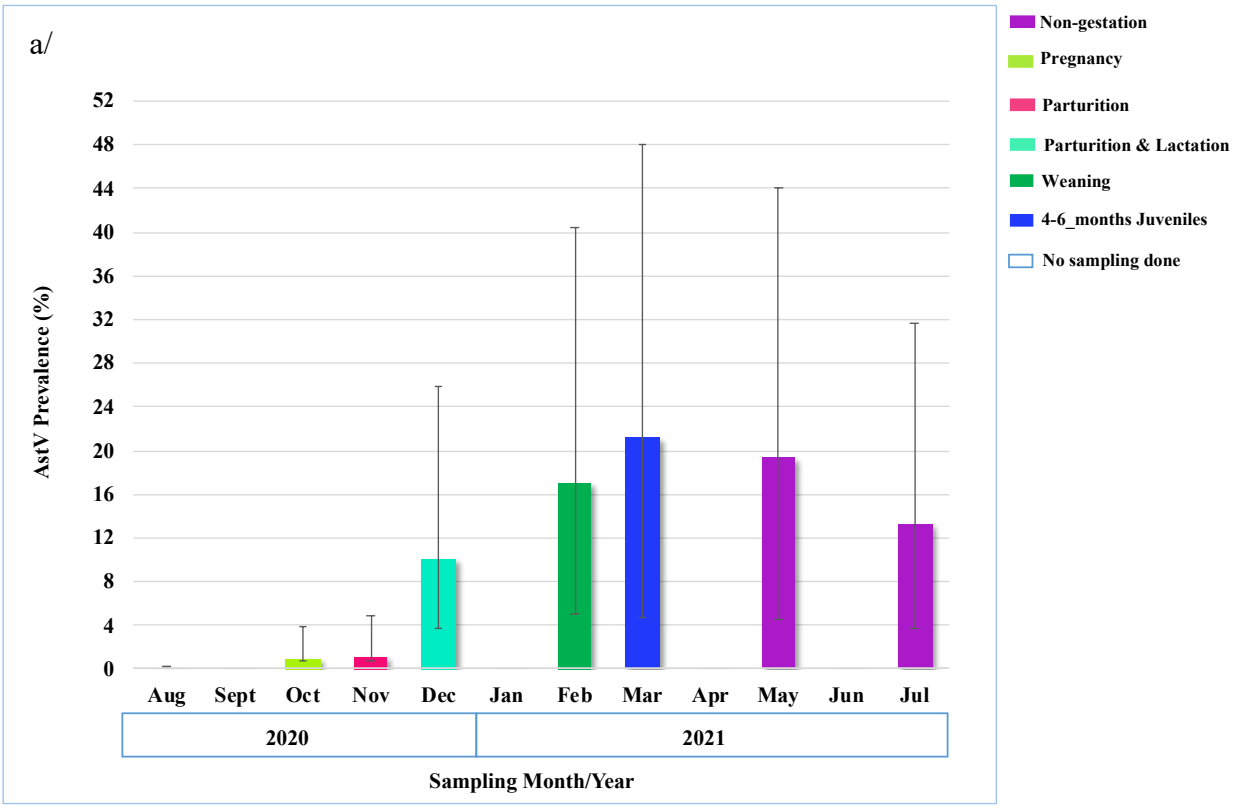
242

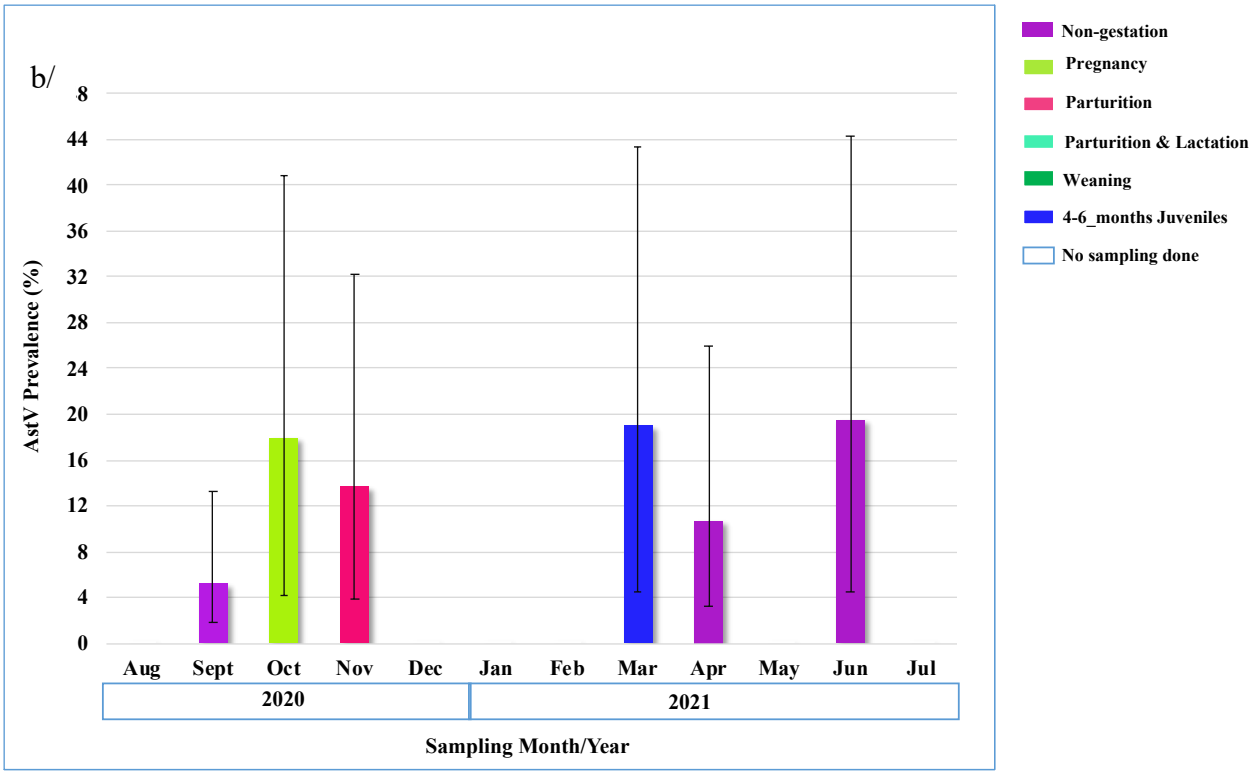
243 ***Prevalence and seasonality of Astroviruses at Chirundu and Magweto site***

244 In total, we analyzed 1559 samples from Magweto cave site, and 1728 samples from Chirundu farm.
245 (Table 2). The overall prevalence of AstVs at bat community level at Chirundu was 10.4% [95% CI:
246 9.0-11.8] (179 positives out of the 1728 samples). The highest prevalence of 21.3% [95% CI: 16.5-
247 26.8] was observed in March 2021 and corresponded to the 4-6 months old juvenile period (Table 2).
248 The prevalence of AstV increased from December 2020 to March 2021, which corresponded to the
249 lactation period, followed by the weaning of four to six months old juveniles. The lowest prevalence
250 was observed in August (0%), October (1% [95% CI: 0.3-2.9]) and November (1.2% [95% CI: 0.4-
251 3.6]), which correspond to the transition between non-gestation and gestation period, as well as the
252 beginning of the parturition period (Table 1, Figure 2a).

Figure 2:

Bat community AstVs prevalence per month at Magweto site. **Fig 2a & 2b** show estimation of the astrovirus prevalence (with CI 95%) at Chirundu (a) and Magweto (b) sites respectively for the months and year sampled. The graphs were plotted with y-axes as the percentage of AstVs prevalence and x-axis as the sampling collection date and the corresponding reproductive cycle stage, each colour code representing a different season.





261 In Magweto site, the overall prevalence of Astroviruses for the insectivorous bat community was 13.7%
262 [95% CI:12.1-15.5] (214 positives out of 1559 samples). There was no clear pattern for the prevalence
263 of AstVs according to reproductive cycle stages in this site (Table 2, Figure 2b). The highest
264 prevalence of 19.4% [95% CI: 14.9-24.9] was observed in June 2021 during the non-gestation period ,
265 and very close prevalences observed during the pregnancy (17.9% [95% CI: 13.7-23]) and 4-6 months
266 old juvenile (19% [95% CI: 14.5-24.4]) periods (Table 2). Lower prevalence was observed in
267 September at the end of the non-gestation period (5.2% [95% CI: 3.3-8]).
268 Results from the GLMM didn't show any effect of bats seasonal reproduction periods on detection
269 of AstV RNA in samples collected at Magweto site (Table 3). For Chirundu site, the GLMM showed
270 significantly higher probability of being positive to astrovirus during the period of weaned 4-6 months
271 old juveniles (odds ratio (OR) = 5.088, 95% CI = 1.33- 36.32, p= 0.016), and significantly lower
272 probability of AstV positivity during the pregnancy period (odds ratio (OR) = 0.136, 95% CI = 0.03-
273 0.81, p=0.006) (Table 3).
274
275 **Table 3*:*** Results of the GLMM with the following explanatory variables for both site (Chirundu and
276 Magweto): PCR_Pan-*Coronavirus*, Pregnancy, Weaning, Weaned juveniles 4 to 6 months. With ref-
277 erences OR odds ratio, CI95 95% confidence interval.
278

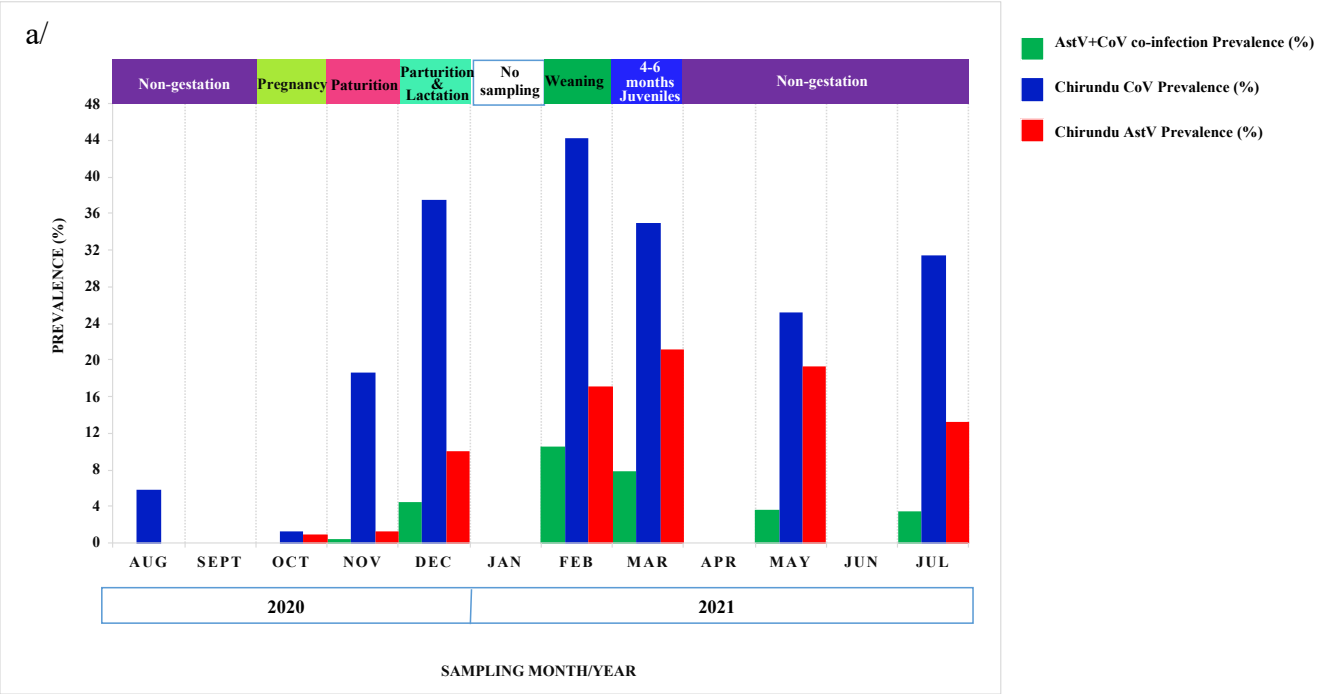
Variables	Odds ratios	p-value	OR CI95
Chirundu Site			
intercept	0.05	6.68e-09	0.010- 0.121
PCR_Pancoronavirus	1.116	0.536	0.785 -1.574
Pregnancy	0.14	0.006	0.027-0.810
Weaning	3.91	0.094	0.721-39.593
Weaned_juveniles 4 to 6 months	5.09	0.016	1.333-36.322
Magweto Site			
intercept	0.235	0.0005	0.088-0.627
PCR_Pancoronavirus	1.238	0.290	0.825-1.825
Pregnancy	0.473	0.145	0.138-1.548
Weaned_juveniles 4 to 6 months	0.678	0.448	0.202-2.256

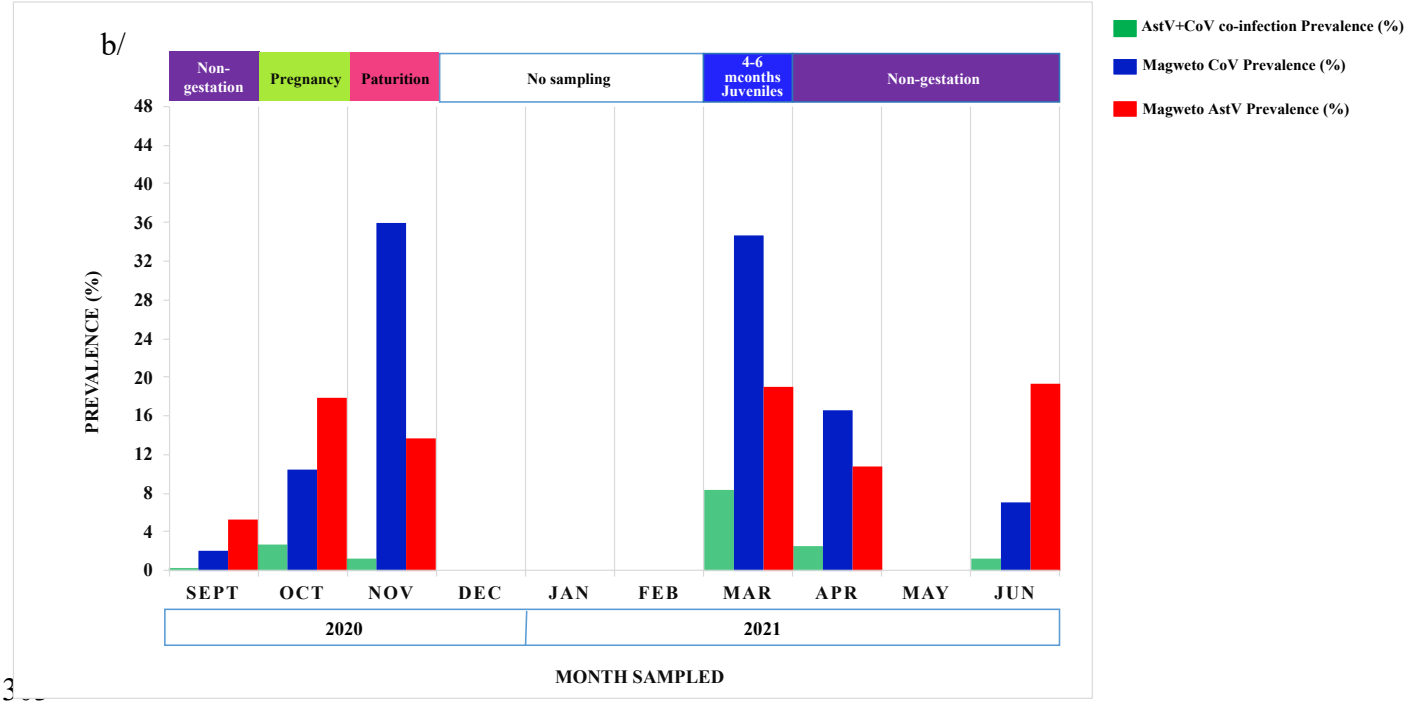
279
280
281 ***Prevalence and seasonality of coinfection with Astroviruses and Coronavirus at Chirundu and***
282 ***Magweto site***

The co-infection prevalence in bat communities from Chirundu and Magweto sites was relatively low. The co-infection prevalence was observed to follow a similar trend as that of CoVs and AstVs prevalences, where it showed peaks that correlated with peaks of the individual viral families (Figure 3a & b). The overall prevalence of co-infection at Chirundu site was 3.6% [95% CI: 3.8-5.8] (Table 2) with the highest co-infection prevalence of 10.6% [95% CI: 6.8-16.1] observed in February 2021 during the weaning period. The lowest prevalences of 0% were observed in August [95% CI: 0.0-0.2] and October [95% CI: 0.0-1.3] during the end of the non-gestation and the pregnancy period, respectively (Table 2, Figure 3a).

The overall prevalence of co-infection at Magweto site was 2.6% [95% CI: 1.8-3.5] (Table 2, Figure 3b). Co-infection in this site was recorded from September 2020 to December 2020 and from March 2021 to June 2021 (Table 2, Figure 3b). The highest prevalence of co-infection was 8.3% [95% CI: 5.4-12.4] in March during the 4-6 months old juvenile period; while the lowest 0.3% [95% CI: 0.05-1.6] was observed in September 2020 during the non-gestation period (Table 2, Figure 3b).

Figure 3*:* Prevalence of *Astrovirus*, *Coronavirus* and co-infections involving both viruses per month: co-infections are represented in green, coronaviruses in blue and astroviruses in red for a/ Chirundu site and b/ Magweto site according to reproduction cycle stages.





Astroviruses genetic diversity

All AstV positive samples (N=423) were sequenced, representing 179 samples from Chirundu, 229 from Magweto, seven from Mabura, seven from Honde valley, and one from Baobab tree. In this study we sequenced all AstVs positive samples from 5 sites: (Chirundu farm N=179; Magweto cave N=229; Mabura cave N=7; Honde Valley N=7 and Baobab tree N=1). Of the 423 samples, 214 (50.6%) were amplified from *Hipposideros* spp., 63 (14.9%) from *Rhinolophus* spp., 53 (12.5%) from *Miniopterus* spp., 10 (2.4%) from *Nycteris* spp., seven (1.7%) from *Eidolon* spp. and for the remaining 76 samples known to originate from bats for which we couldn't determine the genus/species.

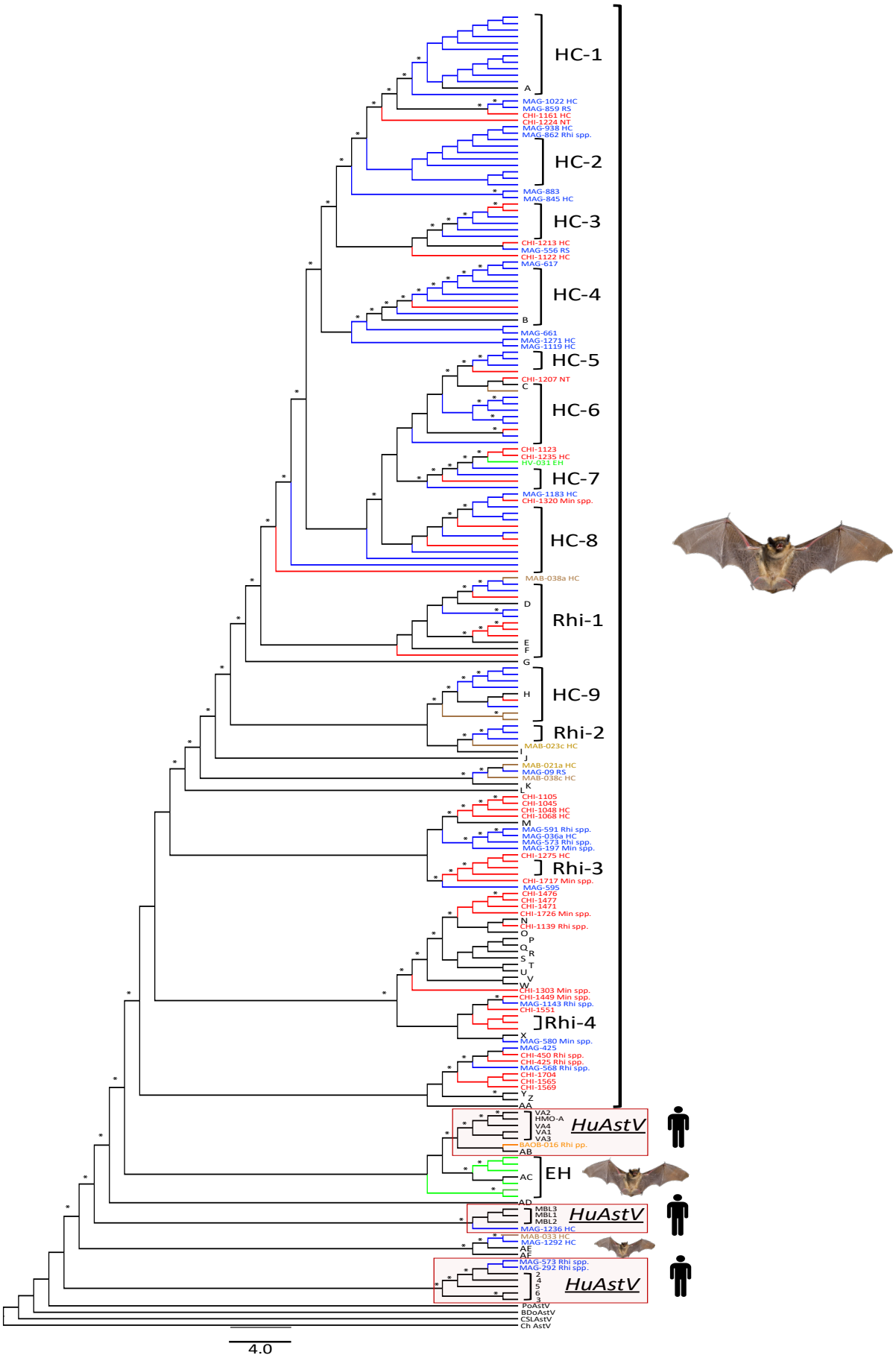
A phylogenetic analysis was conducted with 155 partial sequences generated during this study and representative of the genetic diversity observed. All clustered within the *Mamastrovirus* genus (Figure 4).

Two sequences isolated from Magweto cave, one from *Rhinolophus* spp. (MAG-573) and 1 from *Hipposideros* spp. (MAG-292) clustered with a group of Human astroviruses (*HuAstVs*) (Figure 4). This cluster was well supported (Bootstrap >90%) and the nucleotidic acid identities between Bat Astroviruses MAG-573, MAG-292 and *HuAstV*-2 were 96 and 93% respectively (Data not shown). The other specific cluster of bat astrovirus showing a close relation to *HuAstVs* is a sequence isolated from *Hipposideros* spp. (MAG-1236) in Magweto, it clustered with *HuAstV-MLB-1, -2* and *3* (Figure 4). Six sequences isolated from the frugivorous bats *E. helvum* found in Honde Valley and one from *Rhinolophus* spp. collected in a Baobab tree formed a sister clade with another group of Human astroviruses *HuAstV-VA-1, -2, -3, -4* and *HuAstV-HMO-A* (Figure 4). In this study, the majority of the sequences were closely related to astroviruses identified in insectivorous bats (Figure 4). These were

mainly amplified from *Hipposideros* spp. (HC-1 to -9) and *Rhinolophus* spp. (Rhi-1 to -4) bat species. One cluster comprised specifically of sequences isolated from *Rhinolophus* spp. Most of these sequences were isolated from Chirundu and a few of them from Magweto, and clustered together with AstV strains from *Nyctalus* spp. and *Verpitillio* spp. bat species isolated in Czech Republic (references Y & Z in the phylogenetic tree) (Figure 4). Another cluster comprising of the majority of AstV sequences from Chirundu and some from Magweto site, were characterized from *Miniopterus* spp. and *Rhinolopus* spp. All together, they formed a phylogenetic cluster with strains of bat astroviruses isolated from *Miniopterus* spp. from Madagascar and China (references N, O, R, U, T), *Rhinolophus* spp. from Korea (references P, Q) as well as *Rousettus* spp. and *Paratrianops* spp. Bat AstVs *(BAstV)* from Madagascar (references V, W) (Figure 4)

Two major clades from Chirundu and one from Magweto, isolated from *Miniopterus, Rhinolophus* and *Hipposideros* spp. of bats also phylogenetically clustered with a strain from Mozambique isolated from *Nycteris thebaica* (reference M). One sequence from Magweto isolated from *Rhinolophus* spp. and two from Mabura cave isolated from *Hipposideros* spp., clustered with a strain of bat astrovirus isolated from *Myotis* spp. in Madagascar (references K, L) (Figure 4). A cluster, comprising two clades from Magweto and one sequence from Chirundu and three sequences from Mabura cave isolated from *Rhinolophus* and *Hipposideros,* were closely related to bat astrovirus strains from *Hipposideros* spp. of Mozambique and Laos (references H, I) (Figure 4). The last small group comprising one clade from Chirundu, four sequences from Magweto, all isolated from *Rhinolophus* spp., and one sequence from Mabura isolated from *Hipposideros* spp. clustered together with bat astrovirus strains from *Rhinolophus* spp. of Laos and Korea (references D, E, F) (Figure 4).

One of the large clusters shows the phylogenetic relationship amongst sequences derived from, *Hipposideros* spp. (Clades HC-5 to -8), these making up the majority of the sequences in this clade and one isolated from *Miniopterus* spp., one from *E. helvum* and one from a *Nycteris* spp.. The bat astroviruses in this clade, all showed a close relation to a bat astrovirus strain isolated from *Hipposideros spp.* of Mozambique (reference C). The largest cluster constituted mainly by Magweto site sequences and some Chirundu sequences, from *Hipposideros* spp. (clades HC-1 to 4) and a few *Rhinolophus* and *Nycteris* spp., clustered with a strain from *Hipposideros* spp. of Mozambique (references A, B) (Figure 4).


HC-1
A
MAG-1022 HC
MAG-859 RS
CHI-1161 HC
CHI-1224 NT
MAG-938 HC
MAG-862 Rhi spp.
HC-2
MAG-883
MAG-845 HC
HC-3
CHI-1213 HC
MAG-556 RS
CHI-1122 HC
MAG-617
HC-4
B
MAG-661
MAG-1271 HC
MAG-1119 HC
HC-5
CHI-1207 NT
C
HC-6
CHI-1123
CHI-1235 HC
HV-031 EH
HC-7
MAG-1183 HC
CHI-1320 Min spp.
HC-8
MAB-038a HC
D
Rhi-1
E
F
G
HC-9
H
Rhi-2
MAB-023c HC
I
J
MAB-021a HC
MAG-09 RS
MAB-038c HC
K
L
CHI-1105
CHI-1045
CHI-1048 HC
CHI-1068 HC
M
MAG-591 Rhi spp.
MAG-036a HC
MAG-573 Rhi spp.
MAG-197 Min spp.
CHI-1275 HC
Rhi-3
CHI-1717 Min spp.
MAG-595
CHI-1476
CHI-1477
CHI-1471
CHI-1726 Min spp.
N
CHI-1139 Rhi spp.
O
P
Q
R
S
T
U
V
W
CHI-1303 Min spp.
CHI-1449 Min spp.
MAG-1143 Rhi spp.
CHI-1551
Rhi-4
X
MAG-580 Min spp.
MAG-425
CHI-450 Rhi spp.
CHI-425 Rhi spp.
MAG-568 Rhi spp.
CHI-1704
CHI-1565
CHI-1569
Y
Z
AA
VA2
HMO-A
VA4
VA1
VA3
HuAstV
BAOB-016 Rhi pp.
AB
EH
AC
AD
MBL3
MBL1
MBL2
HuAstV
MAG-1236 HC
MAB-033 HC
MAG-1292 HC
AE
AF
MAG-573 Rhi spp.
MAG-292 Rhi spp.
2
4
5
6
3
HuAstV
PoAstV
BDoAstV
CSLAstV
Ch AstV
4.0

358
359

Figure 4*:* Phylogenetic tree of *Astroviruses* partial *RdRp* gene. The sequences detected at Chirundu site are represented by red branches, at Magweto site by blue branches, at Honde Valley by green branches, at Mabura cave by brown branches and at Baobab tree site by an orange branch. Human *Astrovirus* are highlighted in red rectangles and bold letters represent the different bat AstV references used to build this tree (See Supplementary Data: Figure 4). The tree was built using the maximum likelihood method based on the GTR + G4 + I model. The robustness of nodes was assessed with 1000 bootstrap replicates. Bootstrap values >70 are in asterisk and those <70 are not shown. HC=*Hipposideros caffer*; RS=*Rhinolophus simulator*; NT=*Nycteris thebaica*; EH= *Eidolon helvum*; Min=*Miniopterus* spp.; Rhi=*Rhinolophus* spp.. MAG=Magweto site; CHI=Chirundu site; BAOB= Baobab; MAB= Mabura site; HV=Honde valley site. The scale bare represented the number of substitutions per site. The AstV sequences used as references are detailed in supplementary data: DOI **10.5281/zenodo.8399361**

Discussion

The overall AstVs prevalence was respectively 10.0% and 13.7% for Chirundu and Magweto sites, confirming their circulation in bat populations from Zimbabwe. However, compared to studies in other African countries, the observed prevalence was low: Hoarau et *al* and Lebarbenchon et *al* detected AstV in 20-22% of individual bats in Mozambique and Madagascar, respectively [21,23]. This difference in prevalence of AstV compared to other countries can be attributed to different study designs, longitudinal sampling in our case versus transversal studies in Mozambique and Madagascar [21,23]. Furthermore, these differences could also result from the studied bat species having varying immunological responses and susceptibilities to different viruses [40], and the different localities resulting in subtle changes that may impact the infection dynamics.

We also observed difference between the two main sites studied (Chirundu and Magweto). Furthermore, the differences in prevalence between the two main individual populations can be attributed but not limited to differences between habitats, bat population structures and bat species composition of bat communities and also anthropogenic factors. Therefore there is need for a more comprehensive study, covering several years of follow up, to fully establish the factors contributing to the AstV viral dynamics among bat populations at these two sites.

At Chirundu site increasing RNA-AstV prevalences were observed from the lactation to the 4-6 months old juvenile seasons with 21.2% of RNA-AstV detection during the latter. Astroviruses have been described to display seasonal variations in prevalence [23]. Drexler et *al* observed different peaks of astrovirus detection associated with different stages of the reproductive season with peaks correlating to maternal aggregations during breeding season and after parturition season due to establishment of a susceptible sub-population of weaned new-born bats who did not yet have their own adaptive immunity [24]. In our study the prevalence was high during some of the months compared to overall prevalence, which might be related to the effect of reproductive cycle stages on the shedding of viruses, as demonstrated for other viruses. In another study the reproductive season with juveniles and immature individuals showed a very high prevalence of CoV as compared to prevalences in the absence of juveniles and presence of sexually mature bats thus suggesting different stages of the reproduction having effect on viral shedding [31, 41–43]. In this study we observed a

399 similar trend for AstVs for one of the study sites. Furthermore, Mendenhall et *al*, also identified the
400 bat juvenile stage as exhibiting a greater AstVs viral burden than any other stage of the reproductive
401 season [43]. The range of prevalence observed during the highest peaks coincides with the ≥20%
402 overall AstV detection reported by Hoarau et *al* [21] during the time of their sampling in February
403 and May. Here we observe a peak during the 4-6 months old juvenile season in Chirundu site which
404 corresponds to a high influx of immunologically immature individuals in the bat population, similar
405 to what was observed in the Chidoti et *al*, (2022) for CoV infection [31].
406 Juveniles and immature individuals are known to contribute to shedding pulses of viruses as they
407 develop productive infections during the acute phase [27], which coincides with the waning
408 protection of maternal antibodies [31], thus increasing the susceptibility and rate of infection in the
409 young.
410 We could not observe a clear trend at Magweto site, which might be related to the absence of samples
411 from December 2020 to March 2021, which correspond to the lactation and weaning periods.
412 In our study we also investigated co-infection of bats by coronaviruses and astroviruses. We compared
413 the co-infection prevalence with the prevalence of astroviruses and coronaviruses described at each
414 site during the same reproduction cycle stages. The co-infection prevalence showed a similar trend to
415 that observed in the individual viruses, whereby during observed peaks of high AstVs and CoVs
416 detections, the co-infection also peaked at similar phases of the reproduction cycle stages. Thus the
417 co-infection of bats by both CoVs and AstVs is high when there is high infection of bats by either
418 virus.
419 The overall prevalence of co-infection was 3.5% and 2.6 % in Chirundu and Magweto respectively
420 while the bats from Madagascar and Mozambique respectively showed a AstV-CoV co-infection of
421 5% (±2.7%) and of 1.7% (±1,1%) [9,40]. Co-infection and recombination of viruses in bats have been
422 reported on several occasions including co-infection of bats by coronaviruses and astroviruses
423 [9,40,44]. Bats infected with either CoV or AstV were shown to be more likely co-infected with the
424 respective virus [26]. In our study, no effect of coronavirus infection/shedding on astrovirus
425 infection/shedding was observed.
426 In the current study we detected bat Astroviruses from *Hipposideridae, Rhinolophidae*, *Pteropodidae,*
427 *Nycteridae* and *Miniopteridae* families. Astroviruses are known to show no host restriction and are
428 widespread within the Chiroptera order [16,24]. The majority of the astroviruses amplified were from
429 *Hipposideros* and *Rhinolophus* species and these clustered with AstV sequences derived from
430 Mozambique, Madagascar and China of the same families [21,23,24]. The observed trend is due to
431 the two genera being the most dominant species of bats at both sites where sampling was done.
432 Therefore, the probability of higher detection of AstV in these species is expected and evidently
433 observed to be higher than other rare or less dominant species. Active transmission events of

434 astroviruses amongst *Rhinolophidae* and *Hipposideridae* bat species are known to occur [24]. In this
435 study, a high degree of species-specific tropism, especially in bat astrovirus related clusters was
436 observed with astrovirus strains isolated from *Hipposideros* spp. clustering with each other and
437 similar trends for *Rhinolophus* and *Miniopterus* spp. However, the clusters were not site-specific as
438 sequences from Magweto, Mabura and Chirundu sites formed clusters with each other. We also
439 observed a large phylogenetic cluster which comprised of specific *Hipposideros and Rhinolophus*
440 AstV sub-clades (HC-1 to -8 and Rhi-1 to -4) as well as a few AstV sequences from other bat species
441 (Figure 4). Previous studies on astroviruses in bats have also described this family of viruses to be
442 involved in species tropism. However recent studies challenged this assumption and highlighted the
443 fact that AstV cross-species transmissions are more frequent than previously thought [4,45]. In our
444 study, AstVs sequences isolated from different bat species of the same site clustered together. This
445 indicates potential cross species transmission of astroviruses within each site, as reported by Xiao et
446 *al* in bats sharing the same habitat [45]. We also observed phylogenetic clustering of astroviruses
447 from Chirundu and Magweto sites into same clades showing a great diversity of astroviruses in
448 circulation within the studied bat communities. Novel astroviruses species have been reported in bats
449 hosts [25], and in this study it is evident due to the diverse astroviruses described. The AstV isolated
450 from *E. helvum*, frugivorous bats, clustered together and showed close phylogenetic relation to *BAstV*
451 isolated from *Rousettus* spp. bats in China and *Taphozous* spp. bats in Laos. One strain isolated from
452 *E. helvum* clustered with *Hipposideros* bat astroviruses sequences isolated in Chirundu site. This
453 could mean that frugivorous bats and insectivorous bats can be infected by the same type of AstVs
454 strain thus indicative of the multi-host spectrum for the Astroviridae family. Detection of AstVs in
455 the frugivorous bats was low compared to insectivorous bats, and this can be attributed to the
456 insectivorous bats ability to adapt and harbour more astroviruses than frugivorous bats [45].
457 Two strains, Mag-292 *H. Caffer*, Mag-573 *Rhinolophus* spp. clustered within the *HuAstv* type 1 to 6,
458 that are known to cause infection and diarrhoea in children and infants [1]. Six of our strains isolated
459 from *E. helvum* and one from *Rhinolophus* spp. formed sister clades to *HuAstV* HMO, associated with
460 severe extra-intestinal illnesses in humans [5] and VA-1-4 strains. Another AstV strain detected in
461 *Hipposideros* bat species clustered with *HuAstv* MLB-1, -2 and -3 which are all known to cause
462 gastro-intestinal and neurological diseases with mild-severe symptoms in humans [46]. This
463 phylogenetic clustering could suggest that *HuAstV* and *BAstV* may have a shared common ancestor.
464 However, with limited sequence data the evolutionary history is merely a speculation that needs to be
465 further investigated through full genome analysis. Another hypothesis for the observed relation is that,
466 since bats are known to be important reservoir hosts of astroviruses with great diversity, they could
467 be ancestors of the *HuAstV* due to the high prevalence of AstV detected in bats. *HuAstV* transmission
468 occurs via the faecal-oral route and contaminated food or water [1], therefore it is unlikely that

anthroponosis may have occurred. However, this observed relation could be due to a rare event or mutation, or a new strain of bat astrovirus that has not been described yet. Further full genomic data as well as epidemiological data on these bat colonies are needed to reach a full conclusion.As novel strains and host species continue to be discovered, understanding AstV transmission and their zoonotic potential is essential. Dissemination of *HuAstV* and AstV from zoonotic reservoir hosts remain a significant threat to public health [7].

Conclusion

This study offered important contributions to the understanding of virus diversity in an under-sampled region of the world, and the sequences contribute to a better picture of co-evolution and transmission among bat species. Multiple sampling points as in the study further give a more comprehensive picture of how viruses vary over space and time and host species. Astroviruses have a high genetic diversity, multiple mechanism of generating additional diversity, and infect a wide range of host species therefore understanding their prevalence and infection dynamics in their wildlife hosts can help to predict or prevent the emergence of novel astrovirus strains into domestic animals and human populations.

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Conflicts of Interest: We declare that we have no conflicts of interest

Author's contribution: Conceptualization: VC, MA, HdN, MB, LG and FL.; **Methodology**: VC, MA, LG, HdN, DP, EG, GM, EM and FL.; **Software:** VC, MA, HdN and FL; **Validation**: VC, MA HdN, JC, and FL; **Formal Analysis**: VC, HdN, JC, and FL.; **Investigation**: VC, VP, NC, GM, and FL.; **Resources**: DP, GM, MP, MB, HdN, LG, and FL.; **Data Curation**: VC, HdN, and FL.; **Writing – Original Draft Preparation**: VC and FL; **Writing – Review & Editing**: HdN, MA, JC, MB, LG, VP, EG, DM, DP, EM, GM and FL; **Supervision**: HdN, DP, GM and FL.; **Project Administration**: LG, MB, HdN and FL; **Funding Acquisition**: MB, HdN, LG and FL.

Data, scripts, code, and supplementary information availability

Sampling Data, Astrovirus sequences, Graphs and Figures

https://doi.org/10.5281/zenodo.8399450

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GenBank Accession Numbers

The AstVs sequences have been deposited to the GenBank under the following numbers: OQ271049 - OQ271203

The Cytochrome B sequences have been deposited to the GenBank under the following numbers: OM487705-OM488020

Statistical analysis

The script used for the GLMM analysis is available at: https://doi.org/10.5281/zenodo.7847934

Supplementary data: https://doi.org/10.5281/zenodo.8399361

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