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, collection date ranged 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 ~~the~~ amplified sequences were identified. One group comprised of sequences isolated from *Hipposideros, Rhinolophus* and *E. helvum spp* clustered with Human Astrovirus strains: *HuAstV* types1-6, *HuAstV*-MLB1-3 and *HuAstV*-VA-1. A second group comprised of 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. Coinfection of bats with CoVs and AstVs at Magweto and Chirundu sites was 2.6% and 3.5% respectively.

Introduction

Astrovirus are single-stranded positive sense RNA (+ssRNA) viruses. They are non-enveloped with an icosahedral morphology and a genome length of approximately 6.2 to 7.7 Kb [1]. They infect a wide variety of both domestic and wild marine and terrestrial mammals, including humans (*Mamastrovirus*) as well as avian hosts (*Avastrovirus*) [2]. Recently *Astrovirus* infections in fish and insects have been reported [3,4]. Bats and wild birds are considered natural reservoirs of astroviruses [5]. Astroviruses have been found to occur in over 80 non-human species including a diverse number of bat species from Europe, Africa, America and Asia. Human astroviruses (*HuAstVs*) have been identified as causal agents of acute viral gastrointestinal illness worldwide particularly in children, immunocompromised people and the elderly [3–5]. Beyond this well-known clinical manifestation of *Astrovirus* infections, neurovirulent *Astrovirus* infections have also been reported in both humans and domestic animals [6,7]. To note, in humans, the majority of *Astrovirus*-associated encephalitis or meningitis were reported in immunocompromised people [8–10]. According to the International Committee on Taxonomy of Viruses (https://talk.ictvonline.org), *Mamastrovirus* are classified within 19 recognized species, *MAstV*-1 to -19, and two genogroups GI and GII. All classic and novel Human Astrovirus belong to four different species, *MAstV*-1, *MAstV*-6, *MAstV*-8 and *MastV*-9 [2,11,12]. Astroviruses do not seem to have a common reservoir [12]. However, numerous cross-species transmissions of *Astrovirus* have been documented [4]. More particularly, certain *HuAstV* strains are closely related to rodent, feline, mink, ovine and porcine *Astrovirus* [2,4,12].

To date, no human *AstV* strains have been associated with Bat *AstV*. Bat *Astrovirus* belong to *MAstV*-12, and *MastV*-14 to -19 species. Bat-borne viruses represent an extensive research field owing to the plethora of viruses carried out by the Chiropterans. Bats are known to be persistently infected by astroviruses [13]. This is inclusive of many species of insectivorous bats which harbor these viruses [14]. To date, a high diversity of Bat *Astroviruses* from different bat families has been reported worldwide [9,15]. In Europe a variety of bat species belonging to the Yangochiroptera suborder have been discovered to harbor several astroviruses [16]. In Africa, bat astroviruses were reported in Egypt, Gabon, Madagascar, Kenya and Mozambique [5,15,17–19]. Astroviruses are known to occur in bat species with high prevalence and exceedingly high genetic diversity whereby infections in these hosts are minimally pathogenic [20,21]. Despite all these studies, large gaps still exist to map the extent of existence of these viruses in bats' populations [22].

Astrovirus prevalence and detection in bat colonies seemed rather correlated to abiotic ~~factor~~ factors such as seasons and year of sampling than biotic factors such as sex and reproductive status or yet viral co-infection [23]. Viral shedding in bats occurs in spatial and temporal pulses that can drive spillover to other animals or humans [24] Shedding pulses in bats determine varying degrees in the

prevalence of viruses where if they do not occur, viruses are rarely detected or at low prevalence and when they occur, higher prevalence is detected [24].

In this study we enlarge the spectrum of bat *Astroviruses* knowledge in Africa by identifying and analyzing Bat *Astroviruses* from different bat species colonies according to season in Zimbabwe.

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).

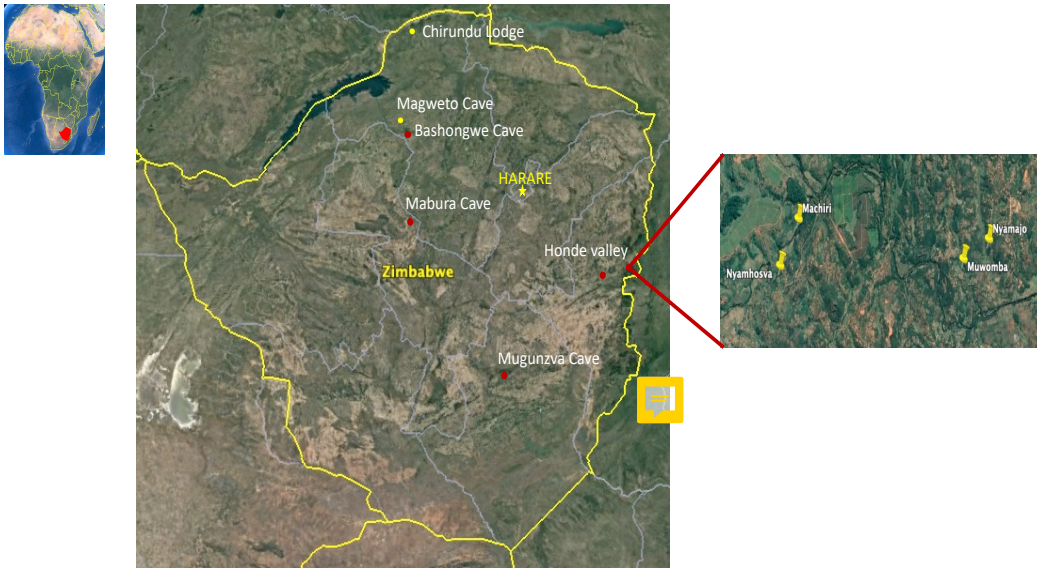


Figure 1: The study sites highlighted on the map show the sampling areas for insectivorous and frugivorous bats colonies across Zimbabwe. Red circles represent community approach study sites; yellow circles represent longitudinal survey sites (individual approach).

Table 1: Number of Astrovirus positives per each site/sampling year in pooled faecal samples from frugivorous and insectivorous bats at different 14 sites.

Sampling Sites	type of roost	Date of collect	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	Baobab	December 202	*Rhinolophus spp.*	21	21	1
Total				**740**	**306**	**16**

All these sites were chosen according to the presence of bat colonies and the existence of anthropic activities. For instance, the selected caves and ancient mine ~~selected~~ are regularly visited by local people to collect bat guano which is used as fertilizer, and to hunt bats for consumption. Frugivorous bats roosting sites in trees were close to maize crops or/and fruit tree cultures.

All sites, except for three were visited at different times during the sampling period (Table 1). The same sampling method was used at all sites every session as previously described [25]. Briefly, two square meters of plastic sheets were laid down at each site/per sampling session, underneath the bat colonies and left overnight. Faeces were collected from each plastic sheet at a rate of ≈ 6 grams of pooled faeces in a 15 ml tube with 6 ml of homemade RNA stabilization solution (*https://protocol-online.org*). Back in the laboratory, samples were stored at -80°C until analysed.

Bat individual sampling

Individual bat samples which had already been collected from two study sites for a study on coronaviruses by Chidoti et *al*. were used in this study (Figure 1) [26]. These two sites, one cave (Magweto) and one building basement (Chirundu Farm) had been visited from August 2021 to July 2022 on a monthly basis (Figure 1, Table 2) [26]. Unfortunately, this study was conducted during the COVID 19 crisis and we were not able to access the study sites every month as planned owing to the

imposed lock downs. Faecal samples had been collected by placing two square meters plastic sheets underneath the bat colonies. Only one faecal dropping (~~clean and~~ not contaminated by other faeces or urine) per 20 cm^2 was collected, assuming it represented one individual. Faeces were conserved individually in a 1.5 ml tube filled up with 0.5 ml of home-made RNA stabilization solution (*https://protocol-online.org/*) and stored at -80°C before further laboratory analyses.

Table 2: Prevalence of Astroviruses and Coinfection with Coronaviruses and confidence intervals (CIs) per month at both Magweto and Chirundu sites in faecal samples from individual sampling of insectivorous bat communities

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)**

Periods of gestation (pregnancy), parturition, lactation, weaning and presence of 4-6 months old juveniles had been determined based on observations (from captures and observation of the roosting bats) combined with literature, as already reported in Chidoti et *al.* [26]. The reproductive season at Chirundu and Magweto site had been observed to begin in September to February for the predominant bat species. The different insectivorous bat families observed at both sites had been found to be synchronous regarding their reproductive cycles, and consensus reproduction periods had been determined based on the literature and observations [26, 27].

Nucleic acid extraction and RT-PCR.

Community samples.

Nucleic acids extraction was done from all faecal samples as previously described [26]. Briefly, biological material (Faeces) of three or four sample tubes collected from the same plastic sheet were pooled and transferred in a 50 ml tube with 20 ml of PBS 1X then vigorously mixed. Tubes were centrifuged at 4500 rpm for 10 min. The supernatant was first filtered using gauze in order to eliminate faecal matter and transferred in fresh tubes then re-centrifuged at 4500 rpm for 10 min. The

supernatant was filtered through a 0.45 µm filter to remove eukaryotic and bacterial sized particles. Seven millilitres of filtered samples were centrifuged at 250,000 g for 2.5 h at 4°C. The pellets were re-suspended in 600 µl H_20 molecular grade and 150 µl were used to extract RNA and DNA using NucleoSpin® RNA Kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's protocol.

Individual samples

Nucleic acids were extracted from 200 µl of faecal samples preserved in 0.5 ml RNA stabilization solution using 5X MagMax Pathogen RNA/DNA Kit (ThermoFisher Scientific, Illkirch-Graffenstaden, France), as already described in [26]. The faeces were vortexed vigorously (30Hz) using Retsch MM400 Tissue lysser for 5 min to fully homogenise and mix the faecal particles, followed by centrifugation at 16000 g for 3 min to fully separate the supernatant from the faecal debris. A volume of 130µl of the supernatant was used for the isolation and purification stage of the nucleic acids using Mag Max extraction kit with the automatic KingFisher Duo Prime Purification System extractor (ThermoFisher Scientific, Illkirch-Graffenstaden, France). A final volume of 80µl of eluted RNA/DNA was stored at -80◦C.

Bat species identification

All positive *Astrovirus* bat species were identified by sequencing *cytochrome b* [28]. *S*equences were then compared to available bat sequences in the GenBank database using *Basic Local Alignment Search Tool* (BLAST) ~~program~~.

Astrovirus detection

Reverse Transcription (RT) was done using random hexamers were done on 5µl of RNA sample template using 1µl random hexamers, 0.5µl Oligo dT primer, 0.4µl of dNTPs (10mM) (ThermoFisher Scientific, Illkirch-Graffenstaden, France) and 5.5µl molecular grade water incubated at 65°C for 5 min. This was followed by addition of 4µl of Buffer 5X, 2µl of 0.1M DTT (M-MLV Reverse Transcriptase, Invitrogen, ThermoFisher Scientific) and 1µl of RNAse OUT, incubated at 37°C for 2 min. A volume of 1µl of M-MLV (M-MLV Reverse Transcriptase, Invitrogen, ThermoFisher Scientific, Illkirch-Graffenstaden, France) reverse transcriptase was added to the mixture followed by incubation at 25ºC for 10 min, 37ºC for 50 min and 70ºC for 15 min. The cDNA obtained was then used to partially amplify the *Astrovirus RNA-dependent-RNA polymerase* gene (*RdRp*) by using a semi-nested Pan-Astrovirus PCR system developed by Chu et *al* [13]. Positive PCR products (422 bp) were gel-agarose purified (Geneclean Turbo Kit, MP Biomedicals, Illkirch-Graffenstaden, France) and then sequenced in both 5' and 3' directions (LGC, Berlin, Germany) by using Sanger method.

For the community approach, purified PCR products were cloned by using Topo PCR Cloning kit according to the manufacturer's protocol (ThermoFisher Scientific, Illkirch-Graffenstaden, France). Ten clones per PCR product were sequenced in both 5' and 3' direction using the Sanger method (Eurofins, Germany).

Phylogenetic analyses

Overlapping sequences were assembled into contiguous sequences using Geneious software package V. 2021.2.2 (Biomatters Ltd, Auckland, New Zealand). Partial non-concatenated nucleic acid sequences of the new *Astrovirus* were aligned using MEGA 7 [29], with minor manual adjustments. Sites that could not be unambiguously aligned and divergent regions were excluded from subsequent analyses. Phylogenies were inferred using Maximum Likelihood (ML) method implemented in PhyML [30]. The suited evolution model was selected by Akaike's Information criterion (AIC) using Topali software [31]. The reliability of branching orders was tested using the bootstrap approach (1000 replicates) [32] and the GTR + F+ I substitution model was determined as the best suited evolution model.

Temporal variations of Astrovirus prevalence and bat reproductive phenology

In the same way as in Chidoti et *al*, the prevalence of astrovirus infection was calculated at the community level for small insectivorous bat species from the two longitudinal study sites, Magweto and Chirundu farm [26]. The proportion of RNA AstV-positive samples were estimated per month and site with 95% confidence intervals (CI) using Wilson score test *(https://epitools.ausvet.com.au/ciproportion)* [33]. A descriptive analysis of AstVs prevalence per month and corresponding reproductive season at each site was done.

The influence of the different phases of the bat reproductive cycle (periods of pregnancy, parturition/lactation, weaning, weaned juveniles of 4 to 6 months old) and of *Coronavirus* infection/shedding on the prevalence of astroviruses was tested by running a generalized linear mixed model (GLMM) for each site (Magweto and Chirundu) as described in Chidoti et *al* [26]. Parturition and lactation were analysed as one season because the observations did not allow the separation of the two periods as they were interlinked. The response variable with a binomial distribution was the *Astrovirus* PCR result of the samples, and the explanatory variables with fixed effects were the different phases of the reproduction cycle. Given *Coronavirus* PCR results on the same samples were available through our previous study (see below), *Coronavirus* status was also integrated into the model as fixed variable to investigate any effect of co-infection on *Astrovirus* infection/shedding. The reproductive phases were coded as 1 if it was during the corresponding reproduction phase and 0 if it

was not. A session identification code was included as a random effect to control for repeated measures from the same sampling session to account for clustered samples collected.

Astrovirus and Coronavirus co-infection

Coronavirus data, produced by Chidoti et *al* [26], on the same sample sets were used in this study to assess the *Astrovirus* and *Coronavirus* co-infection in bat communities from Magweto and Chirundu sites and a descriptive analysis of prevalences was carried out.

Results

Astrovirus detection in pooled sampling sites

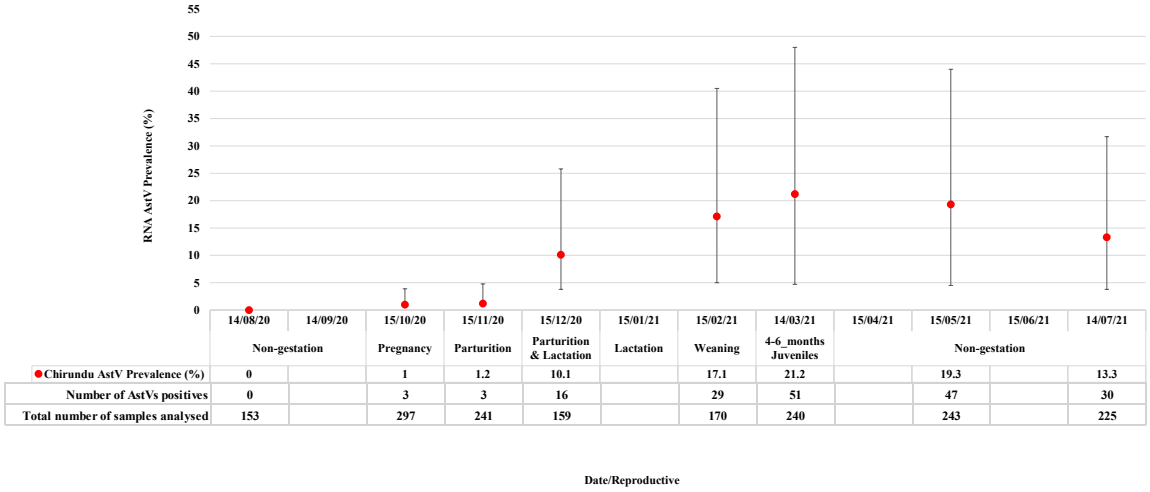
The sampling at these sites varied from June 2016 to December 2020 (Table 1). At Magweto 24 sampled were collected. In Bashongwe collection was carried out in March, July and December 2018, and 251 samples were collected. In Mabura cave site, sampling was done once in 2016 and 2017 and a total of 58 samples were collected. In Mugunza cave site, sampling was done once in 2017 and 50 samples were collected. In the Honde Valley site, 336 samples were collected under 9 *Eidolon helvum* roost trees (Table 1). Five (62.5%) pooled samples from Magweto site were positive for AstVs out of 8 pooled samples analysed and all 72 pooled samples from Bashongwe cave were negative for AstV (Table 1). Six (31.6%) out of 19 pooled samples from Mabura cave tested for AstVs were positive and all of the 13 samples from Mugunza tested for AstVs were negative (Table 1). In Honde Valley, 173 pooled samples were tested for AstVs and only four (2.3%) were positive (Table 1). Only one (4.8%) out of the 21 samples collected in Chirundu Baobab tree site was positive for AstV.

Prevalence and seasonality of Astroviruses at Chirundu and Magweto site

We analysed 1559 samples from insectivorous bats from the Magweto cave site and a total of 1728 faecal samples from bats at the Chirundu farm site (Table 2).The overall prevalence of *Astrovirus* at bat community level of Chirundu was 10.4% [95% CI: 9.0-11.8] (179 positives out of the 1728 samples). The highest prevalence of 21.3% [95% CI: 16.5-26.8] was observed in March 2021 and corresponded to the 4-6 months old juvenile period (Table 2). The prevalence of *AstV* increased from December 2020 to March 2021, which corresponded to the lactation, weaning and 4-6 months juvenile periods. The lowest prevalence was observed in August (0%); October (1% [95% CI: 0.3-2.9]) and November (1.2% [95% CI: 0.4-3.6]), which correspond to the transition between non-gestation and gestation period, as well as the beginning of the parturition period (Table 1, Figure 2a).

Figure 2: Bat community *Astrovirus* prevalence per month at Chirundu site. **Fig 2a & 2b** show estimation of the astrovirus prevalence (with CI 95%) at Chirundu and Magweto sites respectively for the months sampled. The graphs were plotted with y-axes as the number of AstVs positive samples, and the prevalence of AstVs and x-axis as the sampling date and corresponding reproductive season.

a/



	14/08/20	14/09/20	15/10/20	15/11/20	15/12/20	15/01/21	15/02/21	14/03/21	15/04/21	15/05/21	15/06/21	14/07/21
	Non-gestation		Pregnancy	Parturition	Parturition & Lactation	Lactation	Weaning	4-6_months Juveniles		Non-gestation		
Chirundu AstV Prevalence (%)	0		1	1.2	10.1		17.1	21.2		19.3		13.3
Number of AstVs positives	0		3	3	16		29	51		47		30
Total number of samples analysed	153		297	241	159		170	240		243		225

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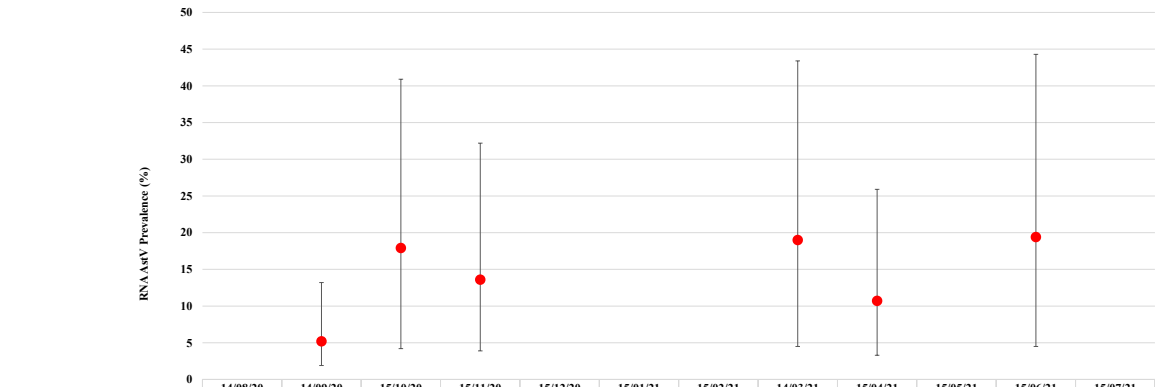

	14/08/20	14/09/20	15/10/20	15/11/20	15/12/20	15/01/21	15/02/21	14/03/21	15/04/21	15/05/21	15/06/21	15/07/21
	Non-gestation		Pregnancy	Parturition	Parturition & Lactation	Lactation	Weaning	4-6_months Juveniles		Non-gestation		
Magweto AstV Prevalence (%)		5.2	17.9	13.6				19	10.7		19.4	
Number of AstVs positives		18	46	31				46	26		47	
Total number of samples analysed		348	257	228				242	242		242	

In Magweto site, the overall prevalence of Astroviruses for the insectivorous bat community was 13.7% [95% CI:12.1-15.5] (214 positives out of 1559 samples). There was no clear pattern for the prevalence

of AstVs according to reproductive seasons in this site (Table 2, Figure 2b).The highest prevalence of 19.4% [95% CI: 14.9-24.9] was observed in June 2021 during the non-gestation period , and very close prevalences observed during the pregnancy (17.9% [95% CI: 13.7-23]) and 4-6 months old juvenile (19% [95% CI: 14.5-24.4]) periods (Table 2). Lower prevalence was observed in September at the end of the non-gestation period (5,2% [95% CI: 3.3-8]).

Results from the GLMM didn't show an effect bats seasonal reproduction periods on detection of RNA-AstVs positive samples at Magweto site (Table 3a). For Chirundu site, the GLMM showed significantly higher probability of being positive to astrovirus during the period of weaned 4-6 months old juveniles (odds ratio (OR) = 5.088, 95% CI = 1.33- 36.32, p= 0.016), and significantly lower probability of astrovirus positivity during the pregnancy period (odds ratio (OR) = 0.136, 95% CI = 0.03- 0.81, p=0.006) (Table 3b).

Table 3*:* Results of the GLMM with the following explanatory variables for both site (Chirundu and Magweto): PCR_Pan-*Coronavirus*, Pregnancy, Weaning, Weaned juveniles 4 to 6 months. With references OR odds ratio, CI95 95% confidence interval.

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

Prevalence and seasonality of coinfection with Astroviruses and Coronavirus at Chirundu and Magweto site.

The prevalence of co-infection of bat communities from Chirundu and Magweto sites was fairly low, however it showed peaks that correlated with peaks in coronavirus and astrovirus infections as observed in the graphs. The overall prevalence of co-infection of insectivorous bats from Chirundu site by Astroviruses and Coronaviruses was 3.6% [95% CI: 3.8-5.8] (Table 2). The highest co-infection prevalence observed in this site was 10.6% [95% CI: 6.8-16.1] in February 2021 during the weaning period while the lowest prevalence of 0% was 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 (Table 2, Figure 3a).

The overall prevalence for co-infection of insectivorous bats in Magweto site by coronaviruses and astroviruses was 2.6% [95% CI: 1.8-3.5] (Table 2, Figure 3b). Co-infection in this site was recorded in all the months, from September 2020 to June 2021, except from December to February during which no sampling took place (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* and *Coronavirus* co-infections (in green), coronaviruses (in blue) and astroviruses (in red) per month in bat communities for a/ Chirundu site and b/ Magweto site.

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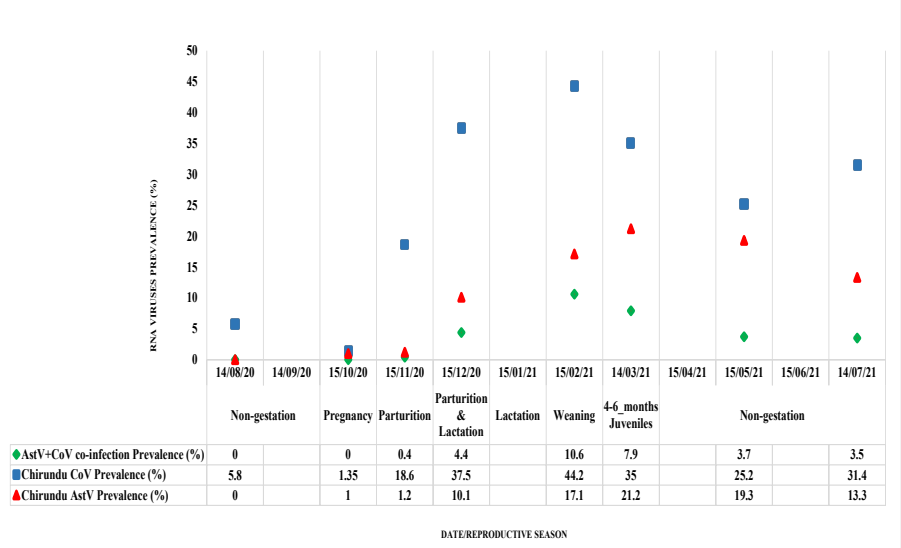

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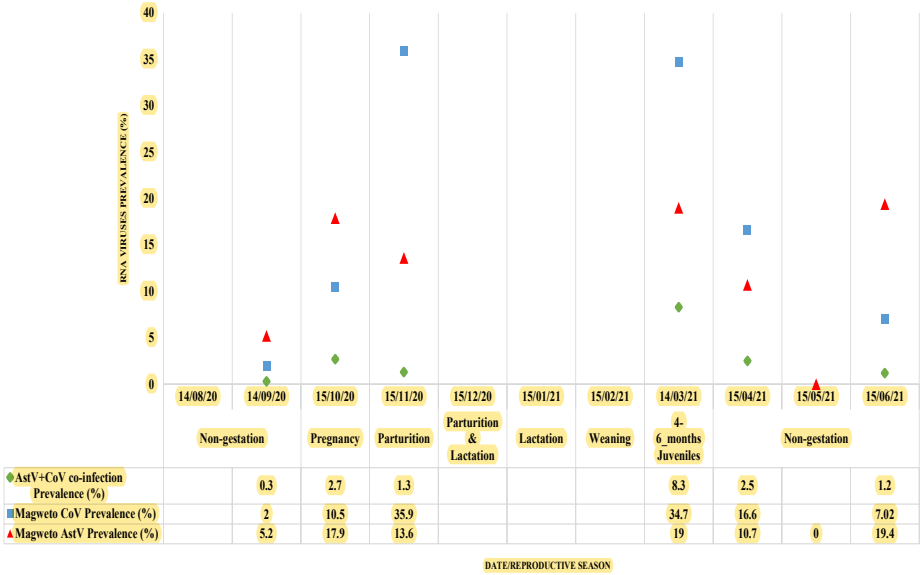
b/



2

300 ***Genetic diversity of RNA Astroviruses***

301 In this study we sequenced all *Astrovirus* positive samples from 5 sites: (Chirundu farm N=179;
302 Magweto cave N=229; Mabura cave N=7; Honde Valley N=7 and Baobab tree N=1). Of the 423
303 samples, 214 (50.6%) were amplified from *Hipposideros* spp, 63 (14.9%) from *Rhinolophus* spp, 53
304 (12.5%) from *Miniopterus*, 10 (2.4%) from *Nycteris* spp, 7 (1.7%) from *Eidolon* spp and for the
305 remaining 76 samples, the bat genus / species could not be determined mainly owing to bad sequence
306 qualities.

307 Phylogenetic analysis of the 155 *RdRp AstV* nucleotide sequences generated for this study showed
308 that they all clustered with the genus *Mamastrovirus* of the *Astroviridae* family (Figure 4).

309 Two sequences isolated from Magweto cave, 1 from *Rhinolophus spp* (MAG-573) and 1 from
310 *Hipposideros spp* (MAG-292) clustered with a group of Human astroviruses (*HuAstVs*) (Figure 4).
311 This cluster was well sustained (Bootstrap >90%) and the nucleotidic acid identities between Bat
312 Astroviruses MAG-573, MAG-292 and HuAstV-2 were 96 and 93% respectively (Data not shown).
313 The other specific cluster of bat astrovirus showing a close relation to human astroviruses, is a
314 sequence isolated from *Hipposideros spp* (MAG-1236) in Magweto, it clustered with *HuAstV-MLB-*
315 *1; -2* and *3* (Figure 4). Six sequences isolated from the frugivorous bats *E.helvum* found in Honde
316 Valley and one from *Rhinolophus spp* collected in a Baobab tree formed a sister clade with another
317 group of Human astroviruses *HuAstV-VA-1;-2;-3;-4* and *HuAstV-HMO-A* (Figure 4). The majority of

the sequences, comprises sequences closely related to insectivorous Bat Astroviruses (Figure 4). The majority of these insectivorous bat related astroviruses sequences were amplified from *Hipposideros* (HC-1 to -9) and *Rhinolophus* (Rhi-1 to -4) bat species. One cluster comprised 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* and *Verpitillio* bat species isolated in Czech Republic (references Y & Z in the phylogenetic tree) (Figure 4). Another cluster comprising of majority of sequences from Chirundu and some from Magweto site, isolated from *Miniopterus* and *Rhinolopus* spp showed phylogenetic clustering 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* and *Paratrianops BAstV* from Madagascar (references V, W) (Figure 4)

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 in by green branches, at Mabura cave by brown branches and at Baobab site by an orange branch. The red rectangle highlighted the phylogenetic relationship between MAG-292, MAG-573 and *HuAstV* strains. 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 species*. MAG=Magweto site; CHI=Chirundu site; BAOB= Baobab; MAB= Mabura site; HV=Honde valley site.

Human *Astrovirus* are highlighted in red and bold letters represented the different bat AstV references used to build this tree:

Bat Astrovirus references:

A - MH013972.1: Hipposideros caffer, Mozambique; **B** - MH013971.1: Hipposideros caffer, Mozambique; **C** - MH013970.1: Hipposideros caffer, Mozambique; **D** - KX858349.1: Rhinolophus spp., Laos; **E** – MC841039.1: Rhinolophus Ferrumquinum, Korea; **F** - MH013989.1: Trianop afer, Mozambique ; **G** - MH013986.1: Trianop afer, Mozambique ; **H** - MH013971.1: Hipposideros caffer, Mozambique ; **I** - KX858371.1: Hipposidoros larvatus, Laos **J** - KY575656.1: Myotis goudoti, Madagascar ;

K - KY575655.1: Myotis goudoti, Madagascar; **L** - MH013988.1: Trianops afer, Mozambique

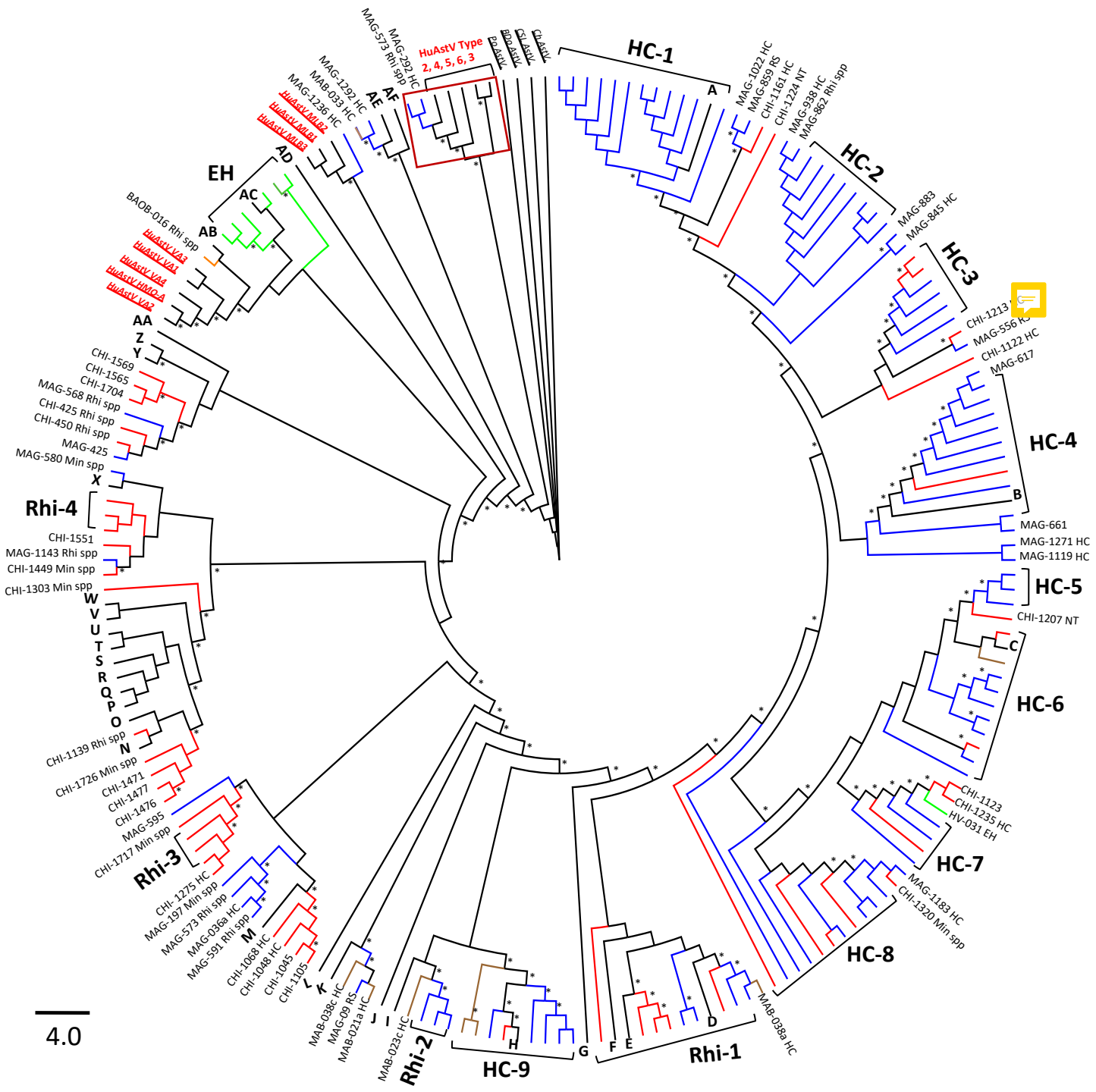
M - MH013076.1: Nycteris thebaica, Mozambique ; **N**- KY575649.1: Miniopterus griveaudi, Madagascar ; **O** - KY575647.1: Miniopterus griveaudi, Madagascar ; **P** - MC840967.1: Rhinolophus ferrumquinum, Korea ; **Q** - MC840969.1: Rhinolophus ferrumquinum, Korea ; **R** - KY575648.1: Miniopterus griveaudi, Madagascar ; **S** - EU847151.1 : Miniopterus magnater, Chine ; **T** - KY575674.1: Miniopterus griveaudi, Madagascar ; **U**- KY575666.1: Rousettus madagascariensis, Madagascar ; **V** - KY575661.1 : Paratrianops furculus, Madagascar ; **W** - KY575644.1 : Miniopterus griveaudi, Madagascar ; **X** - EU847155.1: Miniopterus pussilus, Chine ; **Y**- KP843561.1: Vespertillio Murinus, Czech ; **Z**- KP843558.1: Nyctalus noctule, Czech ; **AA** - KX858513.1: Megaderma lyra, Cambodge ; **AB** - HM368175.1: Myotis myotis, Germany ; **AC** - KX858367.1: Roussetus spp., Laos ; **AD** - FJ571067.1: Taphozous melanopogon, Chine ; **AE** - KX858377.1 : Roussetus spp, Cambodge ; **AF** - KX858378.1 : Roussetus spp, Laos

Human Astrovirus references:

GQ502195.1: Human Astrovirus VA2; JQ673585.1: Human Astrovirus HMO-A; JX857869.1: Human Astrovirus VA4; KJ920196.1: Human Astrovirus VA1; GQ502196.1: Human Astrovirus VA3; JX857870.1: Human Astrovirus MLB3; JQ673581.1: Human Astrovirus MLB1; GQ502188.1: Human Astrovirus MLB2; L23509.1: Human Astrovirus Type 2; AY20891.1: Human Astrovirus Type 4; DQ028633.1: Human Astrovirus Type 5; GQ495608.1: Human Astrovirus Type 6; JF491430.1: Human Astrovirus Type 3

Other Astrovirus references:

GQ914773.1: Porcine Astrovirus (Po AstV); FJ890355.1: Bottlenose Dolphin Astrovirus (BDo AstV); JN 420351.1: California Sea Lion Astrovirus (CSL AstV); æEU669001.1: Chicken Astrovirus (Ch AstV)


HuAstV Type 2, 4, 5, 6, 3
PoAstV
BOVAstV
CSLAstV
ClAstV
HC-1
HC-2
HC-3
HC-4
HC-5
HC-6
HC-7
HC-8
HC-9
Rhi-1
Rhi-2
Rhi-3
Rhi-4
EH
A
AA
AB
AC
AD
AE
AF
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
MAG-1022 HC
MAG-859 RS
CHI-1161 HC
CHI-1224 NT
MAG-938 HC
MAG-862 Rhi spp
MAG-883
MAG-845 HC
CHI-1213
MAG-556 RS
CHI-1122 HC
MAG-617
MAG-661
MAG-1271 HC
MAG-1119 HC
CHI-1207 NT
CHI-1123
CHI-1235 HC
HV-031 EH
MAG-1183 HC
CHI-1320 Min spp
MAB-038a HC
MAB-038c HC
MAG-09 RS
MAB-021a HC
MAB-023c HC
CHI-1275 HC
MAG-197 Min spp
MAG-573 Rhi spp
MAG-036a HC
MAG-591 Rhi spp
CHI-1068 HC
CHI-1048 HC
CHI-1045
CHI-1105
CHI-1139 Rhi spp
CHI-1726 Min spp
CHI-1471
CHI-1477
CHI-1476
MAG-595
CHI-1717 Min spp
CHI-1551
MAG-1143 Rhi spp
CHI-1449 Min spp
CHI-1303 Min spp
CHI-1569
CHI-1565
CHI-1704
MAG-568 Rhi spp
CHI-425 Rhi spp
CHI-450 Rhi spp
MAG-425
MAG-580 Min spp
BAOB-016 Rhi spp
HuAstV VA3
HuAstV VA1
HuAstV VA4
HuAstV HMO-A
HuAstV VA2
MAG-573 Rhi spp
MAG-292 HC
MAG-1292 HC
MAB-033 HC
MAG-1236 HC
HuAstV MLB2
HuAstV MLB1
HuAstV MLB3
4.0

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**).

Discussion

The overall prevalence of *AstVs* in bats was 10% and 13.7% in Chirundu and Magweto sites, indicating a fairly high circulation of this family of viruses in bats in 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 [17,19]. This difference in prevalence of *AstV* compared to other countries can be attributed to the lack of longitudinal survey in the Mozambican and Madagascar studies as sampling effort was done for periods of two separate months in both studies [17,19].

In 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 [17]. 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 subpopulation of weaned new-born bats who did not yet have their own adaptive immunity [34]. 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 seasons on the shedding of viruses, as demonstrated for other viruses. During the reproductive season with juveniles and immature individuals, the prevalence of CoV was very high as compared to prevalence in the absence of juveniles and presence of sexually mature bats [35,36]. In this study we observed a similar trend for AstVs for one of the study sites. Furthermore, Mendenhall et *al*, also identified the bat juvenile stage as exhibiting a greater *Astrovirus* viral burden than any other stage of the reproductive season [37]. The range of prevalence observed during the highest peaks coincides with the ≥20% overall AstV detection reported by Hoarau et *al* [19] during the time of their sampling in February and May. Here we observe a peak during the 4-6 months old juvenile season in Chirundu site which corresponds to a high influx of immunologically immature individuals in the bat population, similar to the what is observed in ~~the~~ Chidoti et *al*, (2022) for CoV infection [26].

During this period, pups are known to contribute to shedding pulses of viruses as they develop productive infections during the acute phase [24], which coincides with the waning protection of maternal antibodies [26], thus increasing the susceptibility and rate of infection in the young.

We could not observe a clear trend in Magweto site, which might be related to the absence of samples from December 2020 to March 2021, which correspond to the lactation and weaning periods.

In our study we also investigated co-infection of bats by coronaviruses and astroviruses. We compared the co-infection prevalence with the prevalence of astroviruses and coronaviruses described at each site during the same reproductive cycles. The co-infection prevalence showed a similar trend to that observed in the individual viruses, whereby during observed peaks of high AstVs and CoVs detections, the co-infection also peaked at similar phases of the reproductive cycle. Thus the co-infection of bats by both CoVs and AstVs is high when there is high infection of bats by either virus. The overall prevalence of co-infection was 3.5% and 2.6 % in Chirundu and Magweto respectively while the bats from Madagascar and Mozambique respectively showed a *AstV-CoV* co-infection of 5% (±2.7%) and of 2.7% (±1,1%) [17,19]. Co-infection and recombination of viruses in bats have been reported on several occasions including co-infection of bats by coronaviruses and astroviruses [17,23]. Bats infected with either *CoV* or *AstV* were shown to be more likely co-infected with the respective virus [23]. In our study, no effect of coronavirus infection/shedding on *Astrovirus* infection/shedding was observed.

In the current study we detected bat Astroviruses from *Hipposideridae, Rhinolophidae*, *Pteropodidae, Nycterida*e and *Miniopterida*e families. Astroviruses are known to show no host restriction and are widespread within the Chiroptera order [13,20]. The majority of the astroviruses amplified were from *Hipposideros* and *Rhinolophus* species and these clustered with *AstV* sequences derived from Mozambique, Madagascar and China of the same families [17,19,20]. The observed trend is due to the two genera being the most dominant species of bats at both sites where sampling was done.

Therefore, the probability of higher detection of *AstV* in these species is expected and evidently observed to be higher than other rare or less dominant species. Active transmission events of astroviruses amongst *Rhinolophidae* and *Hipposideridae* bat species are known to occur [20]. In this study, a high degree of species-specific tropism, especially in bat astrovirus related clusters was observed with astrovirus strains isolated from *Hipposideros spp* clustering with each other and similar trends for *Rhinolophus* and *Miniopterus spp*. However, the clusters were not site-specific as sequences from Magweto, Mabura and Chirundu sites formed clusters with each other. There is a significant phylogenetic clustering of isolated bat astrovirus according to specific species. Previous studies on astroviruses in bats have also described this family of viruses to be involved solely in tropism transmission which is species specific [38–40]. *Astrovirus* sequences isolated from different bat species of the same site clustered together. This indicates potential cross species transmission of astroviruses within each site, as reported by Xiao et *al* in bats sharing the same habitat [40]. We also observed phylogenetic clustering of astroviruses from Chirundu and Magweto into same clades showing a great diversity of astroviruses in circulation within the studied bat communities. Novel astroviruses species have been reported in bats hosts [21], and in this study it is evident due to the diverse astroviruses described. The *AstV* isolated from *E. helvum*, frugivorous bats, clustered together and showed close phylogenetic relation to *BAstV* isolated from *Rousettus spp.* bats in China and *Taphozous spp.* bats in Laos. One strain isolated from *E. helvum* clustered with *Hipposideros* bat astroviruses sequences isolated in Chirundu site. This could mean that frugivorous bats and insectivorous bats can be infected by the same type of AstVs strain thus indicative of the multi-host spectrum for the Astroviridae family. Detection of *Astrovirus* in the frugivorous bats was low compared to insectivorous bats, and this can be attributed to the insectivorous bats ability to adapt and harbour more astroviruses than frugivorous bats [40].

Two strains, Mag-292 *H. Caffer*, Mag-573 *Rhinolophus spp* clustered within the *HuAstv* type 1 to 6, that are known to cause infection and diarrhoea in children and infants [40]. Six of our strains isolated from *E. helvum* and one from *Rhinolophus spp* formed sister clades to Human *AstV* HMO, associated with severe extra-intestinal illnesses in humans [12] and VA-1-4 strains. Another *AstV* strain detected in *Hipposideros* bat species was close and formed a sister clade with *HuAstv* MLB-1, -2 and -3 all known to cause gastro-intestinal and neurological diseases with mild-severe symptoms in humans [41]. This phylogenetic clustering could suggest that *HuAstV* and *BAstV* may have a shared common ancestor. However, with limited sequence data the evolutionary history is merely a speculation that needs to be further investigated through ORFs sequencing and MRCA reading. Another hypothesis for the observed relation is that, since bats are known to be important reservoir hosts of astroviruses with great diversity, they could be ancestors of the *HuAstV* due to the high prevalence of *AstV* detected in bats. *HuAstV* transmission occurs via the faecal-oral route and contaminated food or water [2],

therefore it is unlikely that anthroponosis may have occurred. Third, 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 ~~and true~~ conclusion.

As novel strains and host species continue to be discovered, understanding *AstV* transmission and their zoonotic potential is essential. Disseminating *HuAstV* and *AstV* from zoonotic reservoir hosts remain significant threats to public health [4]. Astroviruses have a high genetic diversity, multiple mechanism of generating additional diversity, and infect a wide range of host species therefore understanding their prevalence 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.7856230

https://doi.org/10.5281/zenodo.7849008

GenBank Accession Numbers

The *Astrovirus* 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

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