

## Characterization and phylogenetic analysis of new bat astroviruses detected in Gabon, Central Africa

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**Summary.** – Astroviruses are emerging RNA viruses that cause enteropathogenic infections in humans and in other mammals. The identification of astroviruses in a wide range of animals highlights the zoonotic importance of these viruses. Bats can harbor many different viruses, among which some are highly pathogenic for humans (for instance, Nipah, Ebola and SARS coronavirus), and also several astroviruses. As some RNA viruses can be directly transmitted from bats to humans, it is crucial to collect data about their frequency, genetic diversity and phylogenetic characterization. In this study, we report the molecular identification of 44 new astroviruses (with a detection rate of 4.5%) in 962 apparently healthy bats that belong to five different species and that were captured in different caves in North-East Gabon, Central Africa. Our results show that bat astroviruses form a group that is genetically distinct from astroviruses infecting other mammals. Moreover, these astroviruses showed an important genetic diversity and low host restriction in bat species.

**Keywords:** bat; astroviruses; Gabon; host restriction; genetic diversity

### Introduction

Astroviruses are members of the *Astroviridae* family and are classified in two genera: *Mamastroviruses* and *Avastroviruses* that infect mammals and birds, respectively ( Méndez *et al.*, 2007; De Benedictis *et al.*, 2011). Astroviruses are small, non-enveloped icosahedral viruses of 28–35 nm in diameter that are characterized by a five- or six-pointed star-like appearance when observed by electron microscopy. These viruses have a positive-strand RNA genome with a poly(A) tail at the 3'end, but no cap at the 5'end. Their genome is about 6,800 to 7,900 nucleotide-long and

is composed of three ORFs (ORF1a, ORF1b and ORF2) that encode, respectively, the protease, RNA-dependent RNA polymerase (RdRp) and capsid precursor protein. Astroviruses are one of the main causative agents of mild to severe gastroenteritis in humans and other mammals (Moser and Schultz-Cherry 2005). Mamastroviruses have been identified in humans suffering from diarrhea, particularly in infants and young children (De Benedictis *et al.*, 2011). Several reports showed that astroviruses are present worldwide and represent the second most important cause of gastroenteritis in children, after rotaviruses (Herrmann *et al.*, 1991; Matsui *et al.*, 2001; Moser and Schultz-Cherry 2005; De Benedictis *et al.*, 2011). Astroviruses have also been identified in several animal species, such as cats, swine, sheep, minks, cheetahs, sea lions, bottlenose dolphins, red deer, dogs, rodents and bats (Woode and Bridger, 1978; Snodgrass *et al.*, 1979; Gray *et al.*, 1980; Williams, 1980; Tzipori *et al.*, 1981; Bridger *et al.*, 1984; Shirai *et al.*, 1985; Woode *et al.*, 1985; Harbour *et al.*, 1987; Marshall

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**Abbreviations:** AstV = astrovirus; CIRMF = Centre International de Recherches Médicales de Franceville; RdRp = RNA-dependent RNA polymerase; RNA = ribonucleic acid; SARS CoV = SARS coronavirus

*et al.*, 1987; Vieler and Herbst, 1995; Englund *et al.*, 2002; Lukashov and Goudsmit, 2002; Zhu *et al.*, 2009; Blomström *et al.*, 2010; Chu *et al.*, 2010; Reuter *et al.*, 2011; Tse *et al.*, 2011). Recently, new astroviruses (MBL1 and VA1) have been identified in humans with diarrhea (Finkbeiner *et al.*, 2008; 2009a; 2009b). Phylogenetic analyses revealed that these new viruses are genetically divergent from all previously described serotypes and that they are related to rodent, mink or ovine astroviruses (Finkbeiner *et al.*, 2008, 2009a,b). These results suggest that astroviruses can infect several different host species and highlight the risk that animals could be reservoirs of astroviruses pathogenic for humans.

Bats (*Chiroptera* order) are considered as the second most abundant, diverse and geographically dispersed vertebrate species after rodents (Calisher *et al.*, 2006; Kasso *et al.*, 2013). These animals can harbor more than 80 virus species and are considered to be the reservoir of many emerging virulent viruses, such as lyssaviruses, Nipah and Hendra viruses, SARS coronavirus (CoV), Ebola virus and Marburg virus. Recent studies highlighted the high prevalence rates and important genetic diversity of enteric astroviruses in bats collected in China, Germany and Hungary (Zhu *et al.*, 2009; Chu *et al.*, 2010; Xiao *et al.*, 2011; Drexler *et al.*, 2012; Hu *et al.*, 2014). In this context, studies on the phylogenetic relationships among astroviruses isolated in wild life animals are the first step to better understand the prevalence, genetic diversity and ecology of these viruses.

Here, we report the detection of new bat astroviruses that circulate in five different bat species in Central Africa (Gabon). Our results show that these bat astroviruses are included in the already described bat astrovirus groups. Moreover, our sequence data suggest that bat astroviruses are characterized by an important genetic diversity and by low host restriction among bat species.

## Materials and Methods

**Study sites and sample collection.** Bats were captured in three caves located in the Belinga mountain complex (Ogooue'-Ivindo province, North-East Gabon): Faucon Cave (1°07'N 13°20' E), Zadié Cave (0°98'N 13°19' E) and Batouala Cave (0°82'N 13°45' E) in July 2009. The distances between the different caves were as follows: 33.7km between the Batouala and Zadié caves, 11.7km between the Zadié and Faucon caves and 37.7km between the Batouala and Faucon caves. Bats were captured with mist nets installed at the cave entrance just before twilight and were individually euthanized. Species identification was done on-site by trained field biologists and their forearm measurements, weight, sex and breeding status were recorded. Samples of different organs (liver, spleen, kidney, lung, heart, intestines, brain and salivary glands) were collected. Samples were frozen and transferred to the Centre International

de Recherches Médicales de Franceville (CIRMF) laboratory (Gabon), where they were stored at -80°C until analysis. Molecular analyses were used to test for the presence of astroviruses in 962 bat intestines, collected in the three caves. Captured bats belonged to four insectivorous species (*Hipposideros cf. ruber*, *Hipposideros gigas*, *Coleura afra* and *Mimiopterus inflatus*) and one frugivorous species (*Rousettus aegyptiacus*).

**Ethical approval.** Bat capture and sampling were conducted with the permissions of the Wildlife and Hunting Department of the Gabonese Ministry of Water and Forestry (N°003/MEFE-PA/SG/DGEF/DCF and N°0021/MEFE-PA/SG/DGEF/DCF). All the capture events, animal handling, euthanasia and sample transfer across country borders were performed in accordance with the guidelines of the American Society of Mammalogists (<http://www.mammalsociety.org/committees/animal-care-and-use>) (Gannon and Sikes 2007).

**RNA extraction.** To save time and money, RNA extracted from pooled intestine samples (five samples/pool) from the same bat species was used. Approximately 100 mg of each intestine sample was pooled and crushed in 500 µl of cold phosphate-buffered saline (Biological Diagnostic Supplies Ltd) at 1500 strokes/min in a ball-mill tissue grinder (Geno/Grinder 2000, Spex CertiPrep) for 2 min. One hundred microliters of this suspension was incubated with 300 µl of lysis buffer from the EZ1 RNA Tissue Mini Kit (Qiagen, Hilden, DE) (Qiagen) for 10 min and RNA was then extracted according to the manufacturer's recommended procedure and eluted in 100 µl of elution buffer.

**RdRp gene amplification by RT-PCR.** Each pool was reverse-transcribed (RT) and amplified by hemi-nested PCR with primers that target part of the RNA-dependent RNA polymerase (RdRp) gene sequence in the astrovirus genome, adapted from Chu *et al.* (2008). Specifically, cDNA synthesis and the first round of PCR amplification were performed using the Superscript III One-step RT-PCR kit (Invitrogen) in a 20 µl reaction mixture that contained 5 µl of pooled bat intestine RNA, 2 µl of the primers (10 µmol/l) PoonFwd1a (5'-GARTTYGATTGGRCKCGKTAYGA-3'), PoonFwd1b (5'-GARTTYGATTGGRCKAGGTAYGA-3') and PoonRev (5'-GGYTTKACCCACATNCCRAA-3'), 1 µl of 1 µg/µl BSA, 0.4 µl of 50 mmol/l MgSO<sub>4</sub> and 1 µl of 1U Taq polymerase. The RT step (50°C for 30 min) was immediately followed by a denaturation step (94°C for 3 min) and 45 PCR cycles (94°C for 15 sec, 50°C for 30 sec and 68°C for 30 sec) and finally by an elongation step (68°C for 10 min). For the second round of PCR amplification, the Platinum Taq DNA polymerase High Fidelity kit (Invitrogen) was used according to the manufacturer's recommendations. Specifically, 3 µl of the first-round PCR product was mixed with 2 µl of each of the primers (10 mmol/l) PoonFwd2a (5'-CGKTAYGATGGKACKATHCC-3'), PoonFwd2b (5'-AGGTAYGATGGKACKATHCC-3') and PoonRev (5'-GGYTTKACCCACATNCCRAA-3'), 5 µl of 10x High Fidelity PCR Buffer (Invitrogen), 1 µl of dNTPs (10 mM of each dNTP), 2.5 µl of 50 mmol/l MgCl<sub>2</sub> and 0.2 µl of 1U Platinum Taq High Fidelity (50 µl of total reaction volume). After an initial denaturation at 94°C for 2 min, 45 cycles of PCR amplification were carried out as

follows: 94°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec and an elongation step at 72°C for 10 min. PCR products (expected amplicon size of 422 bp) were visualized on 2% agarose gels stained with GelRed (Biotium). Positive pools were purified with the QIAquick PCR purification kit (Qiagen) and then sequenced in both directions (SeqLab, Germany). For each positive pool, individual RNAs were extracted from the intestine samples included in that pool, PCR-amplified and positive samples were purified and sent for sequencing, as described above (SeqLab, Germany).

**Statistical and phylogenetic analyses.** Statistical analyses were carried out using the R software (Hothorn and Everitt, 2015). The partial RdRp sequences were compared with a database of complete sequences of almost all available serotypes in GenBank (Supplementary Table 1) to determine whether these sequences were genetically related to any known astrovirus serotype. Phylogenetic analyses were performed to identify the relation of the astroviruses detected in the bat samples with other astroviruses. Multiple alignments were performed using the CLUSTAL W software, implemented in Geneious Pro v.8.1.14 (Biomatters, Auckland, New Zealand, www.geneious.com) (Larkin *et al.*, 2007; Kearse *et al.*, 2012). Phylogenetic inference was assessed using a Bayesian approach. The substitution model HKY85, the nearest neighbor interchange (NNI) branch swapping and 1000 bootstrap replicates were applied, through MrBayes implemented in Geneious Pro v.8.1.14 (Ronquist and Huelsenbeck, 2003; Kearse *et al.*, 2012).

**Nucleotide sequence accession numbers.** All 44 sequences were deposited in GenBank Acc. Nos. KU510441 to KU510484.

## Results

### Characterization of the astroviruses identified in bat intestines

Among the 193 bat intestine RNA pools (192 pools of five samples and one of two samples), the astrovirus RdRp region could be amplified by RT-PCR analysis in 45 pools, which corresponded to an overall detection rate of 4.57% (Table 1). PCR amplification and sequence analysis of the

individual intestine samples included in the positive pools confirmed that 44 samples were infected by astroviruses. The detection rate varied among the five bat species (*H. cf. ruber*, *H. gigas*, *C. afra*, *M. inflatus* and *R. aegyptiacus*): 10.32% for *M. inflatus*, 8% for *C. afra*, 1.23% for *R. aegyptiacus*, 4.31% for *H. cf. ruber* and 3.10% for *H. gigas* (Table 1). The detection rate was significantly higher in *M. inflatus* than in the other three bat species (*R. aegyptiacus*, *H. cf. ruber* and *H. gigas*; *P*-values <0.05).

### Genetic diversity and phylogenetic analysis of bat astroviruses

To investigate the genetic relationships of the 44 astroviruses isolated in bats with those infecting other animals, phylogenetic analyses were performed using the partial RdRp region obtained by RT-PCR amplification (363 to 378bp in relation to the considered sequence) (Fig. 1). All bat astroviruses detected in this study clustered with the bat astroviruses of the *Mamastrovirus* genus, with a bootstrap value of 79% (Fig. 1). Moreover, our astrovirus sequences shared 44.20 to 50.90% of nucleotide identity with turkey astroviruses, 47.90 to 59.50% with human astroviruses and 48.90 to 85.60% within the bat astrovirus group. Phylogenetic analysis showed that all bat astrovirus sequences were genetically divergent from the sequences of astroviruses infecting humans and other mammals (Fig. 1). Within each of the five bat species under study, the genetic variability was important and the percentage of nucleotide identity for the RdRp sequences detected in each species was 57.10–84.50% for *H. gigas*, 55.90–97.80% for *H. cf. ruber*, 62% for *R. aegyptiacus*, 83.60% for *C. afra* and 53.70–98.10% for *M. inflatus*.

The 44 bat astrovirus sequences obtained in this work belonged to five different bat astrovirus groups, of which four are characterized by host restriction within the bat genus (Fig. 1). Among the 16 RdRp sequences detected in *M. inflatus* intestine samples, 15 grouped within the bat astrovirus (AstV) Group 4 *Miniopterus* (56.90 to 85.60% of nucleotides identity) and the other one (09GB424, colored

**Table 1.** Rate of detection of the astrovirus RdRp gene sequence in intestine samples from 962 bats collected in Gabon

Bat family	Bat species	Faucon cave		Zadie cave		Batouala cave		Total	
		N	n (%)	N	n (%)	N	n (%)	N	n (%)
<i>Hipposideridae</i>	<i>Hipposideros cf. ruber</i>	209	1 (0.48)	72	3 (4.17)	113	13 (11.50)	394	17 (4.31)
	<i>Hipposideros gigas</i>	126	5 (3.97)	47	1 (2.13)	53	1 (1.89)	226	7 (3.10)
<i>Emballonuridae</i>	<i>Coleura afra</i>	2	0 (0)	0	///	23	2 (8.70)	25	2 (8.00)
<i>Miniopteridae</i>	<i>Miniopterus inflatus</i>	0	///	0	///	155	16 (10.32)	155	16 (10.32)
<i>Pteropodidae</i>	<i>Rousettus aegyptiacus</i>	0	///	143	1 (0.70)	19	1 (5.26)	162	2 (1.23)
	<b>Total</b>	<b>337</b>	<b>6 (1.78)</b>	<b>262</b>	<b>5 (1.91)</b>	<b>363</b>	<b>33 (9.09)</b>	<b>962</b>	<b>44 (4.57)</b>

The family and species of collected bats as well as the number of samples analyzed (N) and the number of positive astrovirus samples (n (%)) in the three different caves in Gabon are detailed.

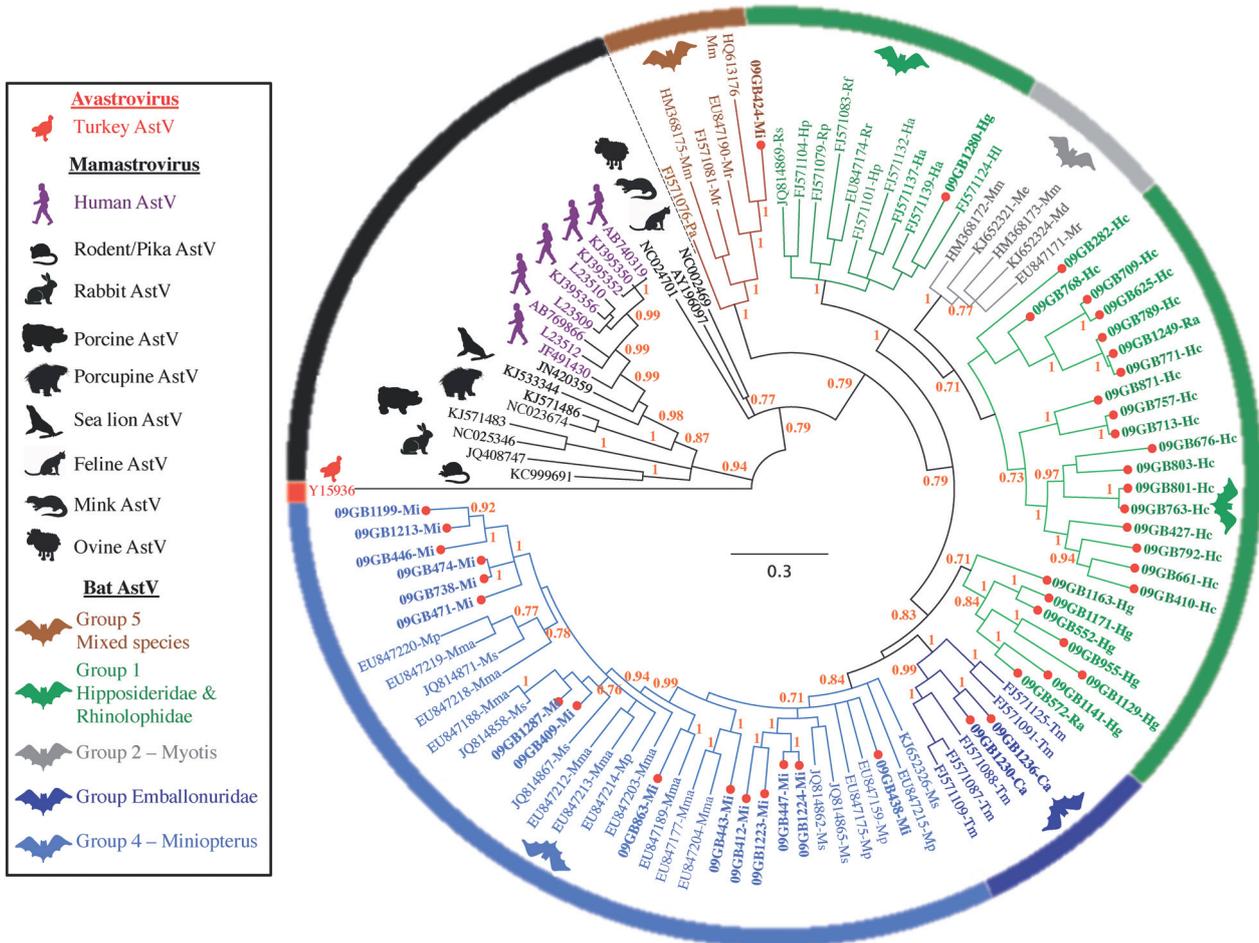


Fig. 1

**Phylogenetic tree constructed using a multiple alignment of the partial RdRp gene sequences detected in bat intestine samples collected in three different caves in Gabon**

Numbers above the branches indicate the maximum likelihood bootstrap values and only values higher than 70 are indicated. A turkey avastrovirus sequence (Y15936) was used to root the tree. The scale bar indicates the number of substitutions per site (0.3). The 44 astrovirus sequences obtained in this study are indicated by red dots and their names are in bold. The bat species is indicated after the GenBank Acc. No. with the following abbreviations: Pa (*Pipistrellus abramus*), Mr (*Myotis ricketti*), Me (*Myotis emarginatus*), Mm (*Myotis myotis*), Md (*Myotis daubentonii*), Sk (*Scotophilus kuhlii*), Ms (*Miniopterus schreiberi*), Mma (*Miniopterus magnater*), Mp (*Miniopterus pusillus*), Rs (*Rhinolophus sinicus*), Rf (*Rhinolophus ferrumequinum*), Rp (*Rhinolophus pearsonii*), Rr (*Rhinolophus rouxii*), Tm (*Taphozous melanopogon*), Ra (*Rousettus aegyptiacus*), Mi (*Miniopterus inflatus*), Hp (*Hipposideros pomona*), Ha (*Hipposideros armiger*), Hl (*Hipposideros larvatus*), Hc (*Hipposideros cf. ruber*), Hg (*Hipposideros gigas*) and Ca (*Coleura afra*). The GenBank Acc. Nos. of the sequences obtained or used in this study are listed in Supplementary Table 1.

in brown in Fig. 1) within the bat AstV Group 5 composed of mixed species (60 to 73.30% of nucleotide identity). The two RdRp sequences detected in *C. afra* (09GB1236 and 09GB1230, colored in dark blue in Fig. 1) were included in a group composed of astroviruses detected in one bat species (*Taphozous melanopogon*) that is part of the *Emballonuridae* family, which includes the *Coleura* genus (68.90 to 74.30% of nucleotides identity). We thus named this group 'Emballonuridae'. All *H. cf. ruber* RdRp sequences (n = 17) clustered together in one group with a bootstrap of 73%. When comparing these *H. cf. ruber* RdRp sequences with

the closest ones obtained from the literature, the nucleotide identity varied from 66.60 to 71%. Six of the seven *H. gigas* RdRp sequences formed a sister cluster with the AstV Groups *Emballonuridae* and *Miniopterus* with a bootstrap value of 71%, whereas the last one (09GB1280) grouped within the bat AstV Group 1 *Hipposideridae* and *Rhinolophidae*. These *H. gigas* RdRp sequences shared with the closest published sequences between 71 and 74.80% of nucleotide identity. The two RdRp sequences detected in *R. aegyptiacus* (09GB572 and 09GB1249) grouped within the two new groups composed of astroviruses detected in *H. cf. ruber* or *H. gigas*

**Table 2. Detection of the astrovirus RdRp gene sequence in the 962 bats in relation to the cave where they were captured**

	Faucon cave	Zadie cave	Batouala cave
Faucon cave	5/262 (1.61)	$P = 0.909$	$P = \mathbf{0.0004}$
Zadie cave		6/337 (1.78)	$P = \mathbf{0.00006}$
Batouala cave			33/363 (9.09)

The number of analyzed samples and the number of positive samples in each cave are indicated (n/N (%)). The chi-square test was used to compare the detection rates in the three caves. Significant  $P$ -values ( $P$ ) are indicated in bold.

(58.90 to 68.20% of nucleotide identity). As the astrovirus RdRp sequences detected in *H. gigas* and *H. cf. ruber* were from the same virus family, all these sequences clustered within the AstV Group 1 *Hipposideridae* and *Rhinolophidae* (Fig. 1). These results confirm that bat astroviruses are characterized by an important genetic diversity, as previously described (Chu *et al.*, 2008; Zhu *et al.*, 2009; Xiao *et al.*, 2011; Drexler *et al.*, 2012; Hu *et al.*, 2014). Moreover, astroviruses do not seem to show host restriction within bat species.

#### *Bat astroviruses do not show geographic structuration*

When considering the astrovirus distribution in the three caves, no significant bat astrovirus phylogenetic clustering within each cave was observed (results not shown in the phylogeny, Fig. 1). Indeed, the viruses isolated in a given bat species could have been from animals collected in any of the three caves with no significant difference (Table 2). Conversely, the detection rate of astrovirus-positive samples was different in the three caves. Specifically, the astrovirus detection rate in the Batouala cave, where all five bat species were captured, was significantly higher (9.09%,  $P$ -value <0.05) than in the Faucon and Zadie caves, where no *M. inflatus* bats were collected (1.78% and 1.61%, respectively) (Table 2).

### Discussion

To improve the current knowledge on bat astrovirus diversity and epidemiology, we investigated five African bat species (*H. cf. ruber*, *H. gigas*, *C. afra*, *M. inflatus* and *R. aegyptiacus*) collected in Gabon. We found that all five bat species carried astroviruses, with variable detection rates (from 10.32% to 1.23%). Moreover, insectivorous (*M. inflatus*, *H. cf. ruber*, *H. gigas* and *C. afra*) as well as frugivorous (*R. aegyptiacus*) bats seem to harbor astroviruses without any host restriction. Our astrovirus detection rate was lower than what reported in previous studies on bats collected in China (Zhu *et al.*, 2009; Chu *et al.*, 2010; Xiao *et al.*, 2011;

Hu *et al.*, 2014). However, in agreement with these studies (Zhu *et al.*, 2009; Xiao *et al.*, 2011), the highest astrovirus detection rate was in bats of the genus *Miniopterus*.

The phylogenetic analysis showed the existence of a distinct phylogenetic sister-group of astroviruses circulating in bats, in agreement with previous works (Zhu *et al.*, 2009; Xiao *et al.*, 2011). This bat AstV group is not included within the currently known diversity of astroviruses circulating in humans (or in other mammals) and forms a divergent group. Our data show that all astrovirus sequences detected in *H. cf. ruber* and six of the seven sequences identified in *H. gigas* intestine samples formed two distinct groups within the AstV Group 1. These two new phylogenetic groups need to be validated as independent from, or still included in the AstV Group 1 by whole genome sequencing. When considering the geographic structuration, no significant phylogenetic clustering of bat astroviruses in the three different caves, where samples were collected, was observed. Indeed, viruses isolated from one bat species could have been from animals captured in any of the three caves. This could be explained by the bat lifestyle, for instance the migration of hundreds of individuals at the same time of the day, thus allowing potential contacts between individuals.

Currently, the species tropism of bat astroviruses is still debated. Some authors suggested that astrovirus transmission and infection have strict host tropism (Baxendale and Mebatsion 2004), whereas others found that bat astroviruses are phylogenetically related to different mammal species (such as minks, ovine and humans) and, therefore, do not show strict host specificity (Xiao *et al.*, 2011). In the present study, among the 44 bat astrovirus sequences we detected, 43 sequences clustered within four bat AstV groups that are characterized by host restriction within the bat genus. This suggests a low degree of host species restriction within the bat species and high capacity of adaption to different bat species. This is in line with the occurrence of multiple cross-species transmission events among different bat species, and probably with other mammal species, that is facilitated by the bat lifestyle (i.e., several bat species living in the same cave), the astrovirus tropism and the oral-fecal transmission of these viruses. On the other hand, one astrovirus detected in *M. inflatus* (09GB424) clustered in the bat AstV Group 5 that includes astroviruses from bat species from different families and is phylogenetically distinct from the other bat AstV groups. This bat AstV group is the only one composed of astroviruses infecting different bat genera and therefore, it could represent the bridge group of bat astroviruses that can adapt to and infect different bat species. Whole genome sequencing analysis of recombination events in bat and other animals' astroviruses should allow confirming this hypothesis.

Genetically, the 44 bat astrovirus sequences (detected in five different bat species) were remarkably diverse (nucleotide sequence similarity between 53.70% and 98.10%).

The high genetic diversity of astroviruses (De Benedictis *et al.*, 2011) associated with the wide variety of host species infected by these viruses suggest and confirm (i) their efficiency in cross-species transmission, (ii) their great adaptability to new environments (hosts) and (iii) their potential zoonotic capability (Lukashov and Goudsmit, 2002). It seems that these viruses evolved in bats for a very long time and therefore, bats could be considered as potential reservoirs of astroviruses.

Finally, unlike Ebola virus, Marburg virus, SARS CoV, Hendra virus or Nipah virus, for which transfers from bats to humans have been documented (Calisher *et al.*, 2006; Han *et al.*, 2015; Marí Saéz *et al.*, 2015), the bat astrovirus group seems to be highly independent from astroviruses that infect other mammalian species, including humans. Indeed, none of the bat RdRp sequences obtained in this and all previous studies belongs to the group of astroviruses that can infect other mammalian species. On the basis of this observation and of the high genetic diversity of bat astroviruses, we hypothesize that these astroviruses are characterized by a very specific host invasion pathway that limits cross-species transfers. This needs to be tested, particularly because the main astrovirus cell receptor has not yet been identified.

In conclusion, this study brings new knowledge on bat astrovirus genetic diversity and epidemiology in Gabon. We molecularly characterized 44 new RdRp sequences from astroviruses that circulate in five different bats species, collected in three caves in North-East Gabon, Central Africa. Our results show that these astroviruses are characterized by a high genetic diversity and low bat species-specificity. Today, emerging and reemerging infectious diseases that affect wild and domestic animals as well as human populations constitute a major public health issue (Wolfe, 2005). In this context, more effort should be focused on fighting newly emerging viruses. One way is to evaluate the diversity of astroviruses circulating in wildlife worldwide, because previous studies focused on restricted geographical areas (Chu *et al.*, 2008; Xiao *et al.*, 2011; Drexler *et al.*, 2012; Hu *et al.*, 2014). Another way is to study recombination events between astrovirus genomes isolated in different host species. Indeed, a major question is whether an astrovirus that naturally circulates in bats (or in another animal species) could recombine with a human variant and create a new variant that can infect another animal population, including humans (De Benedictis *et al.*, 2011).

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**Supplementary information** is available in the online version of the paper.

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