Identification of new astroviruses in synanthropic squamates

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Abstract

Astroviruses have been identified in a wide variety of animal species and are associated with gastro-intestinal disease in humans. Pathologies due to extra-intestinal localization are known in different hosts. We report the detection of astroviruses in synanthropic squamate reptile species (*Podercis siculus* and *Tarentola mauritanica*). Fecal samples were collected from 100 squamates from urban and peri-urban areas of three regions in South Italy and tested for the presence of astroviruses using a broadly reactive (pan-astrovirus) RT-PCR protocol targeting the RNA-dependent RNA polymerase. Astrovirus RNA was detected in 11% of the samples and for six strains a 3 kb-long fragment at the 3’ end of the genome was sequenced, obtaining information on the complete capsid-encoding ORF2 sequence. Viral RNA was also detected in the brain of one of the positive animals. The sequences generated from the astrovirus strains shared low nucleotide identities in the ORF2 (< 43.7%) with other known reptilian astrovirus sequences, hinting to the massive genetic diversity of members of this viral family. Based on the partial RdRp gene of the sequenced strains, however, we observed species-specific patterns, regardless of the geographic origin of the animals, and we also identified a possible inter-species transmission event between geckoes and lizards.

Keywords: astrovirus; squamates; synanthropic reptiles; reptiles

1. Introduction

Astroviruses (AstV, family *Astroviridae*) are small, non-enveloped, single-stranded, positive-sense RNA viruses. Their genome is about 6-7.7 kb in length and is organized in 3-4 open reading frames (ORFs). ORF1a and ORF1b encode the non-structural proteins, and ORF2 encodes the structural proteins (capsid). Some AstV species have a fourth ORF, namely ORFX or ORFY, that encodes a viroporin (Bosch et al., 2014; Lulla and Firth, 2020).

AstVs are currently classified into two genera by the International Committee on Taxonomy of Viruses (ICTV), *Avastrovirus* and *Mamastrovirus*, based on whether they infect avian or mammalian species, respectively (https://ictv.global/report_9th/RNApos/Astroviridae, assessed January 2023). Classification under the genus level has been subjected to revisions and sequence comparison of the capsid-encoding ORF2 has been used to define viral species. A 2011 ICTV proposal calculated ORF2 mean amino acid distances (p-dist) ranges of 0.378-0.750, and 0.006-0.312 between and within groups, respectively for mamastroviruses, and of 0.576-0.742, and 0.204-0.284 between and within groups, respectively, for avastroviruses.

Since then, many putative novel AstVs have been identified in mammals, birds and even in lower vertebrate animal species and invertebrates, challenging the classification system (de Souza et al., 2019; Lu et al., 2022; Shi et al., 2018, 2016; Wohlgemuth et al., 2019). AstVs are associated with gastrointestinal disease in humans and are a common component of the enteric virome of animals (Capozza et al., 2021; de Benedictis et al., 2011; Martella et al., 2020; Wohlgemuth et al., 2019). Also, they may cause fatal hepatitis and systemic infection in avian species (Chen et al., 2020; Todd et al., 2009). Recently AstVs have also been associated to encephalitis and neurological disease in several mammals, including humans (Bouzalas et al., 2014; Matias Ferreyra et al., 2020; Quan et al., 2010).

Many Squamata reptiles, such as snakes, lizards and geckoes, have become synanthropic due to urbanization and their presence is very common in both urban and peri-urban areas. In the Mediterranean basin, these species are represented mainly by geckoes (i.e. *Tarentola* spp.) and lacertid lizards (i.e. *Podarcis* spp.) (Bonardi et al., 2022; Çiçek et al., 2017). Moreover, the use of such reptiles as non-conventional pets, has increased the interactions with humans, also increasing the potential risk for the transmission of zoonotic diseases (Mendoza-Roldan et al., 2020; Mitchell, 2011). Although these animals are highly diffused in households, limited information is available about their virome.

AstVs in lower vertebrates, including some reptile species, have been discovered very recently in meta-transcriptomic studies that have unveiled intricated evolutionary relationships with other classified and unclassified AstVs (de Souza et al., 2019; Shi et al., 2018; Simbula et al., 2019). Inter-species transmission of AstVs has been described and/or hypothesized in several studies (Meliopoulos et al., 2014; Donato and Vijaykrishna, 2017; Mihalov-Kovács et al., 2017; Roach and Langlois, 2021), including reptile AstVs. In this study, we describe the AstVs circulating in the most representative squamate reptile species in urban and peri-urban areas from southern regions of Italy.

2. Materials and Methods

2.1 Sample collection

A total of 100 stool samples were collected from a population of different species of Squamata reptiles (*Podarcis siculus* (n = 63), *Podarcis filfolensis* (n = 6), *Hierophis carbonarius* (n = 2), *Tarentola mauritanica* (n = 11), *Testudo hermanii* (n = 3), *Chalcides ocellatus* (n = 12), *Python molurus* (n = 3) during a study aiming to assess their role as reservoirs of zoonotic parasites, such as Leishmania spp., conducted between 2020 and 2021 in southern Italy (Mendoza-Roldan et al., 2022). In that study, reptiles were captured by hand or by lassoing, from urban/peri-urban areas and dog shelters from three different regions of south Italy; Apulia (n = 56), Calabria (n = 4) and Sicily (n = 40), following convenience sampling, and were transported to the Department of Veterinary Medicine, University of Bari, Italy. Species of reptiles were identified using reference keys (Arnold, 2002), and captured animals were physically examined to assess their health status. Data regarding species, biological state, sex, region, and area in which the animals were sampled, were registered for each animal. Captured animals were humanely euthanized according to established protocols (Warren, 2014) and dissected. Intestine, feces, brain, heart, kidneys, liver, lungs, spleen, and skeletal muscle were individually collected and stored at −20 °C.

2.2 Nucleic acid extraction and screening for AstV

Fecal and organ tissue samples were homogenized in 10% Dulbecco’s modified Eagle’s medium (DMEM) and then centrifuged at 10,000 × g for 3 min. Nucleic acids were extracted from 200 µL of the supernatants using the IndiSpin® Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany), according to the manufacturer’s instructions and stored at −80°C until use.

Screening for AstVs was conducted using a nested reverse transcription (RT)-PCR protocol with a broadly reactive set (pan-AstV) of primers targeting the ORF1b region that is suitable to amplify the majority of known human and animal AstVs with minor modifications(Capozza et al., 2021; Chu et al., 2008)(Chu et al., 2008; Capozza et al., 2022). PCR amplicons were purified and sent for direct sequencing to Eurofins Genomics laboratories (Germany). Sequences of approximately 400 nucleotides (nt) were obtained and analyzed using the web-based tools BLAST (Basic Logic Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed January 2023) and FASTA (https://www.ebi.ac.uk/Tools/sss/fasta/, accessed January 2023), using the default values to find homologous hits.

2.3 Sequence analysis of the ORF1b and ORF2 genes

A 3’ rapid amplification of cDNA ends (RACE)-PCR protocol was used to generate a 3.2 kb amplicon encompassing the 3’ end of the ORF1b, the full-length ORF2, and the 3’ untranslated region (UTR) through the poly-A tail. Briefly, cDNA was synthetized using SuperScript III First-Strand cDNA synthesis kit (Thermo Fisher Scientific, USA) with the reverse primer QT (Scotto-Lavino et al., 2007). PCR was performed using Takara LaTaq polymerase (TaKaRa Bio Europe S.A.S, France) with the AstV ORF1b forward primers (Chu et al., 2008)and the reverse primer QT. The amplicons were purified using PureLink Quick Gel Extraction Kit (Thermo Fisher Scientific, USA), and genomic DNA libraries were obtained using the Nextera XT DNA Sample Prep Kit (Illumina, USA). The libraries were normalized as suggested by the manufacturer's protocol and sequencing was performed on the Illumina MiSeq instrument (Illumina, USA), using a MiSeq reagent kit v2.

De novo assembly and genome annotation were carried out using SPAdes assembler 3.15.2 and Find ORFs, respectively, both implemented in the Geneious Prime software version v. 2021.2.2 (Biomatters, New Zealand).

2.4 Sequence and phylogenetic analyses

Sequence analysis was conducted for both short (~ 400 nt) and long (~ 3.2 kb) fragments of the AstV strains generated in this study. Genome sequences of the complete ORF2 from 23 cognate AstV strains were retrieved from GenBank. Sequence alignment was performed using the Multiple Alignment using Fast Fourier Transform (MAFFT) program version 1.4.0 (Katoh et al., 2002) implemented in the Geneious Prime software v. 2021.2.2. The correct substitution model to perform phylogenetic analyses and evaluation of selection pressure on coding sequences were obtained using MEGA-X version 10.0.5 software. Phylogenetic analysis of the partial ORF1b region and the complete ORF2 region of AstV was conducted using MEGA-X version 10.0.5 software (Kumar et al., 2018).

Maximum-likelihood method, Tamura-Nei 4-parameter model, a discrete gamma distribution and a proportion of invariant sites, 6 categories and 1000 replicates as statistical support were used for the phylogeny of ORF1b sequences. Maximum likelihood method, the General Time Reversible model with a gamma distribution and invariant sites, 6 categories and bootstrapping up to 1000 replicates were used for ORF2 sequences.

2.5 Data availability

Nucleotide sequences of strains ITA/RI62, ITA/RI66, ITA/RI111, ITA/RI112, ITA/RI170 and ITA/RI172 generated in this study and used for phylogeny were deposited in GenBank under accession nos. OQ300505 - OQ300510, respectively. The rest of the data that support the findings of this study are available from the corresponding author upon request.

3. Results

3.1 Molecular screening

Overall, a total of 11/100 (11%) stool samples tested positive with pan-AstV nested RT-PCR screening. In detail, most animals were adult (sexually mature) (79%, n = 79/100), male (61%, n = 61/100), and were collected from Apulia region, Italy (56%, n = 56/100). All animals tested in the present study either lived in urban/peri-urban areas including dog shelters or were housed as pets. When considering the species, AstV RNA was found in 6/63 (9.5%) reptiles belonging to the species *Podarcis siculus* and in 5/11 (45.5%) reptiles belonging to the species *Tarentola mauritanica*, whilst the rest of the animals tested negative. AstV RNA was detected in 6/61 (9.8%) male and 5/39 (12.8%) female individuals. When considering the age, AstV RNA was found in 8/79 (10.1%) adult (sexually mature) and 3/21 (14.3%) young (sexually immature) squamate reptiles. Finally, when considering the region where the animals were captured, AstV-positive individuals were detected in 6/56 (10.7%) from Apulia, 2/4 (50.0%) from Calabria, and 3/40 (7.5%) from Sicily (Table 1 – Suppl. Fig.1).

For 8/11 of the AstV-positive animals, brain samples were also available and screened by pan-AstV nested RT-PCR protocol and one (sample ITA/RI111) tested positive.

For 6/11 AstV-positive squamate reptiles, a 3.2 kb fragment encompassing the complete ORF2 and partial ORF1b genomic regions was generated using a 3’RACE PCR protocol (Table 1).

3.2 Sequence and phylogenetic analyses

Among the samples testing positive for astroviral RNA, 10/11 (90.9%) generated sequences of good quality. Sequence analysis of the 420 bp long fragments of AstV ORF1b genomic region revealed that the strains generated in this study shared 57.4 – 100 % nt and 63.7-100 % amino acid (aa) identity to each other (Table 2). Moreover, the strains ITA/RI111, ITA/RI112, ITA/RI113 and ITA/RI153 shared 73.1 – 77.3% nt identity with an AstV found in a Theobald's toad-headed agama (*Phrynocephalus theobaldi*) (GenBank accession no. MZ375142). The strains ITA/RI62, ITA/RI66, ITA/RI109, ITA/RI142 and ITA/RI172 shared 69.9 – 74.2% nt identity to a Hainan gekko similignum (*Gekko similignum*) AstV (GenBank accession no. MG599917). Finally, the Italian strain ITA/RI170 was 70.5% nt similar to a Hainan oriental leaf-toed gecko (*Hemidactylus bowringii*) AstV (GenBank accession no. MG599918).

On complete ORF2 sequence analysis (Suppl. Table), the Italian strains shared 41.8 – 97.7 % nt and 29.3 – 99.1% aa (Table 3) (Fig.1) identity to each other. Furthermore, the Italian AstV strains were up to 43.7% similar at the nt level and up to 42.7% similar at the aa level to other AstVs identified in squamate reptiles in China (Shi et al., 2018). More in detail, the Italian strains ITA/RI111 and ITA/RI112 identified in geckoes in Apulia, ITA/RI66 identified in a lizard in Sicily, and ITA/RI170 identified in a lizard in Calabria, shared 38.5 – 43.2% nt and 30.4 – 42.9% aa identity with a Hainan gekko similignum (*Gekko similignum*) AstV strain (GenBank accession no. MG599917) found in China. The strains ITA/RI62 (Sicily) and ITA/RI172 (Calabria) identified in lizards were 39.2% and 40.1% nt and 32.4% and 32.2% aa similar to an amphibia AstV, namely Zhejiang Chinese fire belly newt astrovirus 2 (GenBank accession no. MG599916), found in a Chinese fire belly newt (*Cynops orientalis*), respectively.

Based on the partial ORF1b sequences, phylogenetic analysis revealed species-specific patterns, regardless of the geographic origin (Fig.2). However, a unique ORF1b sequence (strain ITA/RI153), identified in a lizard, segregated along with cognate sequences identified in geckoes. Overall, the strains sequenced in this study formed four distinct clusters in the ORF1b-based phylogeny (Fig.2). On phylogenetic analysis of the complete ORF2 genomic region, the six strains sequenced in this study formed three distinct clusters (Fig.1). The gecko AstV strains ITA/RI111 and ITA/RI112 rooted with Chinese gecko AstVs. The lizard AstV strains ITA/RI62 (Sicily) and ITA/RI172 (Calabria) clustered together. Likewise, the lizard strains ITA/RI66 (Sicily) and ITA/RI170 (Calabria), formed a distinct cluster, regardless of the geographic origin (Fig.1).

4. Discussion

The development of pan-viral primers at the family, sub-family or genus level is a powerful tool to generate information on viruses, since the screening of large sample collections can be carried out with a cost-effective strategy. However, this approach comes with several compromises, such as the lower sensitivity of consensus/degenerated primers and the possibility to miss some highly divergent viruses. Moreover, this approach cannot be applied to all virus families, due to their massive genetic diversification hindering primers design. The vast diversity of AstVs in humans and animals also poses challenges for the diagnostics. A pan-AstV RT-PCR protocol was first described in 2008 (Chu et al., 2008) to investigate the circulation of AstVs in bats and since then, it has been cited/used in nearly two hundred studies. This protocol amplifies a 422-nt-long portion of the RdRp (ORF1b) with a nested RT-PCR strategy to improve the sensitivity and it has been successfully applied to generate information from several domestic and wildlife animals (Amoroso et al., 2018; Martella et al., 2020; van Brussel et al., 2020).

In this study, we investigated the presence of AstVs using the aforementioned strategy in a collection of samples obtained from seven different Squamata reptile species, captured in Southern Italy. AstV RNA was successfully detected in 11% (11/100) of the animals. The AstV-positive animals belonged to two different species, *Podarcis siculus* and *Tarentola mauritanica*, whilst the rest of the animal species tested negative. A limit of our study was the convenience sampling used to capture the reptiles. The heterogenous composition of the sample collection did not allow to perform further statistical analysis and evaluate if factors, such as age, sex, animal species, and geographic area, are correlated with infection with AstV. However, it was somewhat unexpected that we were not able to generate AstV-specific amplicons from the other investigated species, likely hinting to a lesser susceptibility to these viruses, to limits of the pan-AstV RT-PCR protocol or to epidemiological/ecological factors.

Sequence data was generated for the majority of strains identified in this study, revealing the high diversity of the AstVs circulating among these reptiles, as reported in previous studies (Lu et al., 2022; Shi et al., 2018). On sequence analysis of the partial ORF1b genomic region, the Italian strains were 47.6% to 99.7% nt similar to each other and exhibited 34.3% to 72.7% nt identity to cognate reptilian and amphibian AstV sequences retrieved from the databases. On phylogenetic analysis, the Italian AstV strains formed four distinct clusters that segregated with Chinese Squamata reptile AstV strains, but also with an AstV strain identified in the amphibian species *Cynops orientalis* (GenBank accession no. MG599916). Interestingly, in our investigation similar AstVs were identified in the different geographic locations where samples were collected, a pattern suggestive of species-specificity or similar dietary habits, rather than a mere environmental exposure. Informative data on the capsid gene was obtained only for six strains. The sequence of the six AstV strains displayed from 41.8% to 97.7% nt and from 29.3% to 99.1% aa identity among each other, whilst they were only up to 43.7% nt and 42.7% aa similar to cognate AstV sequences found in the databases. Phylogenetic analysis of the complete ORF2 genomic region revealed that the Italian reptile AstV strains formed three distinct clusters. All in all, the high heterogeneity observed in the complete capsid-coding region (ORF2) among the Italian strains might indicate the existence of at least four new AstV species, although the ICTV demarcation criteria for AstV genus and species have been defined on the basis of *Mamastrovirus* and

Avastrovirus sequences and an update of classification would be required (Wohlgemuth et al., 2019).

Since we mainly tested fecal samples of Squamata reptiles, we were not able to evaluate whether the identified viruses were able to infect and replicate actively in these animals, or they were instead transferred passively through the gastrointestinal tract, reflecting a dietary and/or environmental contamination.

Nevertheless, we were able to detect the virus in the brain tissues of an AstV-positive animal (sample ITA/RI111). On sequence analysis the AstV-positive brain sample shared 100% nt identity with the fecal sample of the same animal, thus implying that the virus might be able to infect cells of extra-intestinal tissues. This is of particular interest because AstVs have been associated with neurological disease and encephalitis in humans and various animals such as minks, alpaca, cattle, and pigs (Wildi and Seuberlich, 2021). Also, a large meta-transcriptomic investigation for RNA viruses in lower vertebrates, including species of the classes Agnatha (jawless fish), Chondrichthyes (cartilaginous fish), Actinopterygii (ray-finned fish), Amphibia (frogs, salamanders and caecilians) and Reptilia (snakes, lizards and turtles) has identified AstV sequences in the gill, liver, lung and gut of several animals (Shi et al., 2018), thus suggesting that infection of cells of extra-intestinal tissues by AstV is not uncommon in such animals.

The Squamata reptile species collected and examined in this study are considered synanthropic, and their presence is very common in urban and peri-urban areas, interacting with human and domestic animal populations (Simbula et al., 2019). In particular, the two Squamata species, *Podarcis siculus* and *Tarentola mauritanica*, in which we detected AstV RNA, are found on a large scale throughout the Mediterranean basin. This sympatric symbiosis of reptiles, humans and domestic animals may play a role in the transmission of viruses among them by predation, direct, or indirect contact, like in the case of some parasites (Mendoza-Roldan et al., 2020).

To our knowledge, this is the first study describing the identification of AstVs in the synanthropic Squamata reptile species *Podarcis siculus* and *Tarentola mauritanica*. Other Squamata reptile species reported to harbour AstVs include Hainan oriental leaf-toad gecko (*Hemidactylus bowringii*), Guangxi changeable lizard (*Calotes versicolor*) and Guangdong snake species (e.g. *Myrrophis chinensis*) endemic in China (Shi et al., 2018). It is worth noting that the Guangdong snake species makes part of the food culture in some Chinese regions, remarking the importance of studying more in depth the virome of reptilian hosts. A possible example of interspecies transmission of AstV from amphibia to non-human primates has already been described in a wild rhesus monkey in China (Xin et al., 2019). Even though, AstVs have long been considered to infect their hosts in a species-specific manner, lately possible examples of cross-species transmission and recombination events between AstVs from humans and animals have been identified (de Benedictis et al., 2011; Roach and Langlois, 2021). Avian-related AstVs have been identified in the stools of African children with mild-to-severe diarrhoea (Meyer et al., 2015), and in minks in China (Sun et al., 2014), whilst serological investigations have identified antibodies to avian AstVs in poultry workers (Meliopoulos et al., 2014), suggesting that inter-species transmission may occur even between distantly related animal taxa.

The current Covid-19 pandemic has overbearingly re-focused attention on animal viruses with zoonotic potential, as a possible source of threats not only for human health but also for social stability and global economy. Gathering data on the virome of wildlife animals is pivotal to generate a comprehensive database of virus diversity in domesticated and wild animals, useful to assess possible risks for public health and, at the same time, possible threats to animal welfare and conservation.

Ethical Statement

Lizard collection and sampling were authorized by the Ministry for Environment, Land and Sea Protection of Italy (approval number 0073267/2019), the Societas Herpetologica Italica and the Istituto Superiore per la Protezione e la Ricerca Ambientale (approval number 71216).

Conflict of interest

The authors declare no conflict of interest.

Funding

This research was supported by EU funding within the MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

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Table legends

Table 1. List of astrovirus-positive samples identified in this study. The “+” indicates the samples that were amplified in 3’RACE PCR protocol. The “*” indicates the sample that the brain was also positive for astrovirus.

Table 2. Nucleotide (nt) and amino acid (aa) identities (expressed as percentage) of the partial ORF1b genomic region among the AstV strains detected in this study.

Table 3. Nucleotide (nt) and amino acid (aa) identities (expressed as percentage) of the complete ORF2 genomic region among AstV strains detected in this study.

Figure legends

Figure 1. Phylogenetic tree based on the complete ORF2 genomic region of AstV. Bootstrap values greater than 75% were indicated. Black arrows indicate strains detected in this study. Numbers of nucleotide substitutions are indicated by the scale bar.

Figure 2. Phylogenetic tree based on the partial ORF1b sequence (328nt) of AstV. Bootstrap values greater than 75% were indicated. Black arrows indicate strains detected in this study. Numbers of nucleotide substitutions are indicated by the scale bar.