Environmental Surveillance of Human Enteroviruses in Córdoba city, Argentina: prevalence and detection of serotypes from 2009 to 2014

--Manuscript Draft--

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Córdoba, March 15th 2019

Albert Bosch, Ph.D.

Editor-in-Chief

Food and Environmental Virology

Re: Manuscript FAEV-D-17-00002R2

"Environmental Surveillance of Human Enteroviruses in Córdoba city, Argentina:

prevalence and detection of serotypes from 2009 to 2014".

Dear Albert Bosch,

We would like to acknowledge that our article had been considered for publication in the journal Food and Environmental Virology.

We have received your letter with the reviewer's comments for the improvement of our manuscript. We have modified the paper according to their suggestions and hope that all the concerns have been appropriately addressed. All the authors agree with the modifications.

We have included a point-by-point list for your consideration.

Reviewer #1

Page 5, line 6: Why only E14 sequences were submitted to GenBank, what about the others sequences from others genotypes?

A typing error was fixed.

Page 5, line6: If EV sequences from 2013 to 2014 were that obtained by Farias et al. 2017, it should be mentioned in the text.

We included a sentenced to clarify the correspondence of the sequences (Page 5, lines 1-4).

"The novel nucleotide sequences reported in the present study were submitted to GenBank under accession numbers MK435311 to MK435346. Regarding the isolates from 2013-2014 included in the analyses, they correspond to those previously reported in Farias et al. (2018)."

Page 5, line 17: Figure 1: I suggest to change this phylogenetic tree in order to see bootstrap values and clustering of the Argentinean sequences. Remove references sequences (or collapse some of them) other than the genotypes detected in order to make a more clear tree where bootstrap is visible.

As the reviewer suggested, Figure 1 was modified to improve the visualization of Argentinean sequences and detected serotypes.

All the remaining reviewer's suggestions were made.

Reviewer #2

1) Page 7, line 7, the authors mention that AFPs were detected last year and reported by (Perez et al. 2017); but in this work only D68 serotype was detected not E-14. Please remove the sentence or be more explicative about it.

In this sentence we mentioned that the surveillance of AFP was only D68, but that we must bear in mind that there are reports of AFP by E14 .We explain more, following the reviewer's suggestion.

2) The authors reported in the past some studies with part of the same set of samples? please cite your previous works and make a comment.

We included a sentenced to clarify the correspondence of the sequences (Page 5, lines 1-4).

"The novel nucleotide sequences reported in the present study were submitted to GenBank under accession numbers MK435311 to MK435346. Regarding the isolates from 2013-2014 included in the analyses, they correspond to those previously reported in Farias et al. (2018)."

3) figure number 3 have to be just one graphic with a distinctive bars for each serotype.

Reviewer's suggestions were made.

4) This reviewer can observe that 2009 was the year with less positive samples, can be that the samples were not in optimal conditions and they get degraded?

Regarding what the reviewer observed, it is possible that the smallest number of positives for the year 2009 is due to the sensitivity of the technique or to each particular sample. All samples were taken and stored in the same way.

We included a sentenced whit this observation (page 5, line 34)

All the authors have read and approved the revised version of the manuscript.

Looking forward to hearing a positive outcome, we thank you in advance.

Sincerely,

Dr. Adrián Farías

Title Page

Title

Environmental Surveillance of Human Enteroviruses in Córdoba city, Argentina:

prevalence and detection of serotypes from 2009 to 2014.

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Runing title: Prevalence of EVs in sewage of Córdoba city, Argentina from 2009 to 2014.

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Abstract

Environmental surveillance is an effective approach to investigate the circulation of human enteroviruses (EVs) in the population. EVs excreted by patients who present diverse clinical syndromes can remain infectious in the environment for several weeks, and limited data on circulating environmental EVs are available. A six year (2009-2014) surveillance study was conducted to detect non-polio enteroviruses (NPEVs) in the urban sewage of Cordoba city, Argentina. Echovirus 6 (E-6) was the most prevalent (28%), followed by E-14 (17%), E-16 (14%), Coxsackievirus (CV) A9 (11%), E-20 (9%) and CVA24 (6%). Other minority serotypes (E-7, E-13, E-21, E-25 and CVB4) were found, which together represented 14% of the total. In the absence of a systematic EV disease surveillance system, the detection and characterization of sewage-borne NPEVs will help us better understand the changes in EV disease trends and the epidemic background of circulating EVs, which could help interpret the EV trends and warn of future outbreaks in this area.

Introduction

Most treated wastewater, as well as untreated sewage, flow into the environmental water bodies and harbors the potential to impact agricultural, recreational and drinking related water uses. Currently, bacterial indicators, such as total and fecal coliforms, are still commonly employed as the only bioindicator to verify the microbial quality of water (WHO, 2003), specially in South America. EVs belong to the genus *Enterovirus,* family *Picornaviridae* and comprise more than 100 serotypes which are classified into 4 species, *EV-A* to *EV-D*. EVs usually cause silent infection, but sometimes they are associated with serious diseases, such as acute flaccid paralysis (AFP), aseptic meningitis and encephalitis, acute myocarditis, acute hemorrhagic conjunctivitis (AHC), and hand, foot, and mouth disease (HFMD) (Pallansch and Roos, 2007). The monitoring of sewage treatment plants may prove a suitable approach for the study of circulating viruses and the persistence of such viruses in treated effluent. Although environmental surveillance has served primarily as part of Poliovirus (PV) surveillance in many regions of the world, gradually more focus has been put on the circulation and molecular characterization of NPEVs (Vecchia et al., 2012; Pellegrinelli et al., 2013). Many studies have found that, despite the treatment applied, enteric viruses may persist in high levels in wastewaters (Sedmak et al., 2003; Prado et al., 2014; Zheng et al., 2013), proposing the use of a viral indicator of treated sewage contamination. In our group, some previous studies have been carried out for this purpose (Barril et al., 2015; Farias et al., 2018), nevertheless, this is the first carried out for a long time. In Argentina, information on the circulating EVs is limited due to the absence of a specific enterovirus surveillance system. Surveillance based on human specimens is limited and mainly includes testing of specimens collected through AFP and occasional

testing of patients with meningitis or encephalitis. So, despite the increasingly detailed information on temporal/geographical circulation and molecular epidemiology of EVs from various parts of the world, substantial geographical gaps remain in Argentina.

In this report, we present an overview of serotype distribution and temporal dynamics of EVs from environmental surveillance between 2009 and 2014 in Córdoba, Argentina.

Materials and Methods

Environmental molecular survey was conducted in sewage samples (SW) from Córdoba city, the second most populated city of Argentina. A total of 70 samples were collected monthly since January 2009 to November 2014, from the main pipe that enters to the treatment plant. That represents the sewage discharges from about 61% of the population. The samples were transferred from the repository of samples from the laboratory of viral gastroenteritis of the Institute of Virology "Dr JM Vanella". For each sample, 1,500 mL of water were collected and concentrated as previously described by Barril et al. (2015). Enterovirus detection was performed by RT-PCR targeting the 5´ NC region; positive samples were amplified in the VP1 region (Casas et al., 1997; 2001). Amplified products from VP1 region were direct sequenced (Macrogen Inc.). Initial identification of the genomic sequences obtained was carried out by comparing them with all available sequences in GenBank using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The serotype of isolates was determined by the analysis of nucleotide similarity, using the enterovirus automatic genotyping tool (Kroneman et al., 2011) and confirmed by phylogeny. Phylogenetic analyses were performed using sequences obtained in this study and enteroviruses serotypes A (EV-A), B (EV-B) and C (EV-C) prototype sequences. The novel nucleotide sequences reported in the present study were submitted to GenBank under

accession numbers MK435311 to MK435346. Regarding the isolates from 2013-2014 included in the analyses, they correspond to those previously reported in Farias et al. (2018).

Results

During the analyzed period, 50 positive samples were obtained (71%, 50/70) by PCR-5'NC, detecting EV in almost all the months studied. 70% of them (35/50) were identified based on the molecular typing by sequencing a partial region of VP1 gene, identifying 11 different serotypes (Fig.1). Echovirus 6 (E-6) was the most prevalent (28%), followed by E-14 (17%), E- 16 (14%), Coxsackievirus (CV) A9 (11%), E-20 (9%) and CVA24 (6%). Other minority serotypes (E-7, E-13, E-21, E-25 and CVB4) were also found, which together represented 14% of the total (Table 1). Of the total detected, eleven samples were untypable EVs. Seasonality was found for some of the EVs were detected: CVA24 and E-6 were found mostly in winter and summer, E-20 in summer and E-14 in the months of autumn to spring of 2014. In this way EVs is detected during all the seasons of the year (Fig.2). Even though the total numbers of EVs isolated from sewage each year were similar there here were few detections in 2009 and an increase in the year 2014.The distribution patterns of serotypes also varied over the years. The circulation pattern of each NPEV serotype also differed (Fig. 3).

Discussion

Since virus infections have been linked to outbreaks of waterborne diseases, the environmental surveillance constitutes an efficient tool for estimating the extent and the duration of enteric virus circulation in a population (Sedmark et al., 2003; Pinto et al., 2007; Kargar et al., 2009; Pellegrinelli et al., 2013; Wang et al., 2014). This is particularly

important in urban populations with absent or questionable surveillance, when persistent virus circulation is suspected or frequent virus re-introduction can occurs (Farias et al., 2018). Existing research information on environmental samples worldwide has revealed the presence of NPEV strains, but there are only few samples which have been partially or fully sequenced (Zheng et al., 2013; Kyriakopoulou et al., 2015). In this study, we evidenced a continuous NPEVs circulation during a six year period in the central region of Argentina, and a large diversity of serotypes were detected in environmental samples. Six more prevalent circulating EV serotypes were identified in Córdoba, which were E-6, E-14, E-16, CV A9, E-20 and CVA24. In contrast, other minority serotypes (E-7, E-13, E-21, E-25 and CVB4) were only occasionally detected in sewage samples. Sewage may act as a possible reservoir of EVs in Córdoba city and its detection evidence excretion of these viruses from the urban population. This environmental viral presence could represent a significant route of transmission for local populations. This leads to human feces from sewage being the major source of EVs in the environment, spreading to other environmental water matrixes like lakes, or rivers. Currently, no surveillance system is set up to monitor NPEV clinical infection in Argentina. Because most people infected with EVs do not show clinical symptoms, or show only mild symptoms, clinical data on NPEV infection are very limited and mainly from testing of patients with meningitis or meningoencephalitis. Previous studies performed in clinical samples from the Province of Cordoba revealed that the most prevalent serotype was E30, followed by CA9 and CB4 (Farias et al., 2011). In relation to these last two serotypes, the detection occurred a few years before and in different months, with respect to this study demonstrating a history of clinical association in our region with such serotypes. E-6 was the serotype most predominant in sewage in Cordoba, and had a periodicity every two years.

Correspondingly, outbreaks of E-6 associated aseptic meningitis and HFMD were reported in China (Zhang et al., 2013). E-14 is a rarely detected viral serotype, it has been found both in healthy individuals and patients with AFP, as well as in fecal and environmental samples mainly in India, China, and Australia (Chen et al ., 2017). An unusual increase of cases of AFP has been documented in the Metropolitan region of Buenos Aires, Argentina (Perez et al., 2017). Therefore, this data should take into account E14 in the surveillance of AFP. However, symptomatic clinical cases were not reported in Córdoba during the studied period. There was no grouping based on chronologic or geographical criteria. When analyzing the months of detection for each serotype, seasonality was found for some of them: CVA24, E-20, E-14, and E-6. Outbreaks of AHC by CVA24 have been reported in Brazil, and are a notifiable disease in that country (Medina et al., 2016). With our study we can infer its circulation, although we do not have a dimension of its real circulation in the population, in the absence of case reports. So, the low detection number in some serotypes and the untypable EVs might due to the low activity in the surveillance period or is just the result of low copy numbers in the excreta of infected individuals. Further investigation and surveillance are needed to clarify this. The data provided during this study evidenced EVs circulation in Córdoba continuously during the six years studied, showing a great diversity of serotypes. Some of them presented seasonal circulation, epidemiological data relevant to the clinical diagnosis. Despite the large circulation of EV registered and some pathologies produced by these agents, are notifiable in our country, such as meningitis, there are few reports of clinical cases and typing. Due to this, and the existence of subclinical cases, environmental monitoring is a very useful tool for monitoring viruses in our environment.

Compliance with Ethical Standards

Funding sources

Competing interest

On behalf of all authors, the corresponding author (Dr Adrian A. Farias) states that there is no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author Contributions:

AAF conceived and designed the experiments, LNM, FSF, and JJA, performed the experiments and analyzed the data. VEP, GM and SVN contributed materials/analysis tools. AAF wrote the paper.

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Figure Legend

Figure 1: Maximum likelihood phylogenetic tree obtained from the analysis of VP1 region partial sequences obtained from environmental samples from the Province of Córdoba (colored in blue) and prototypic sequences from Enterovirus obtained from the GenBank database. The numbers at each node correspond to bootstrap values obtained with 1000 pseudoreplicates.
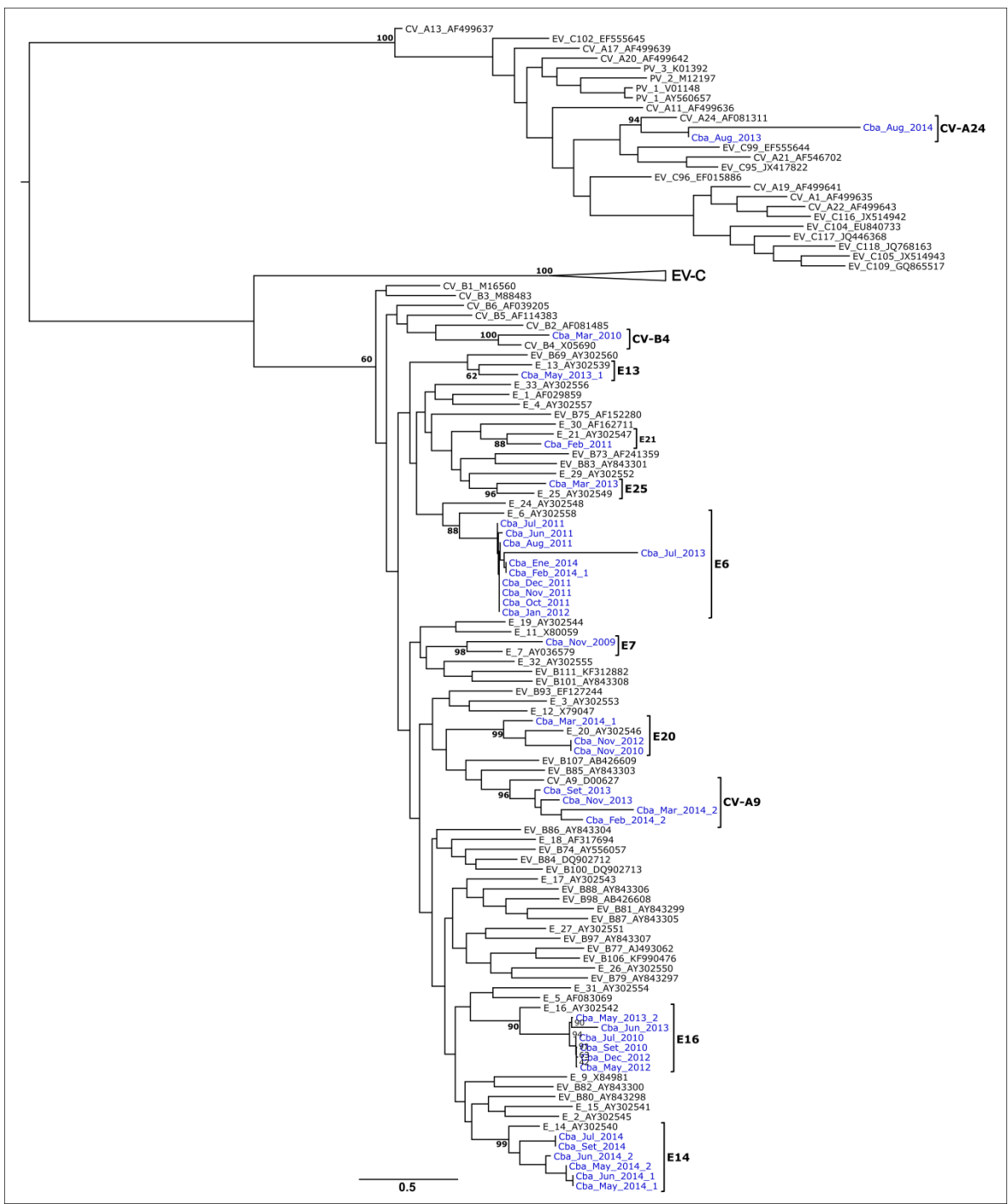
Figure 2: Circulation over time of positive EVs from sewage treatment plant in Córdoba city, during 2009-2014.
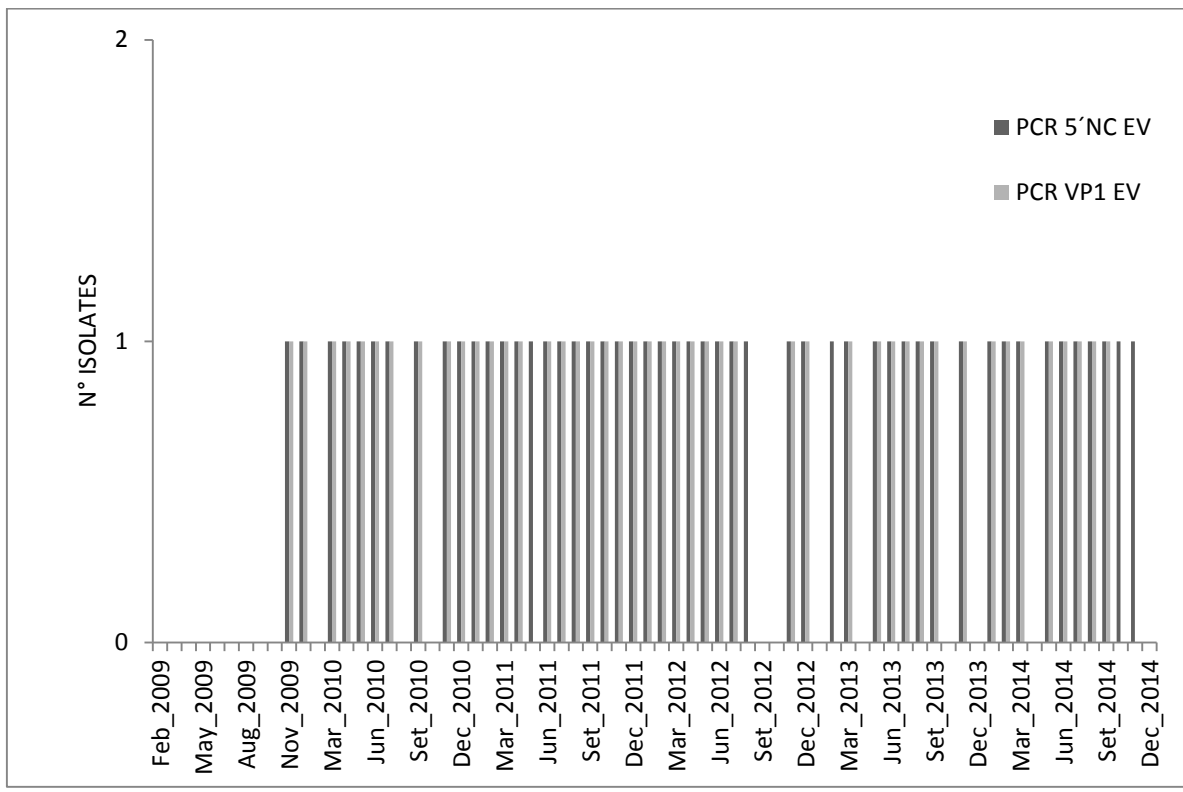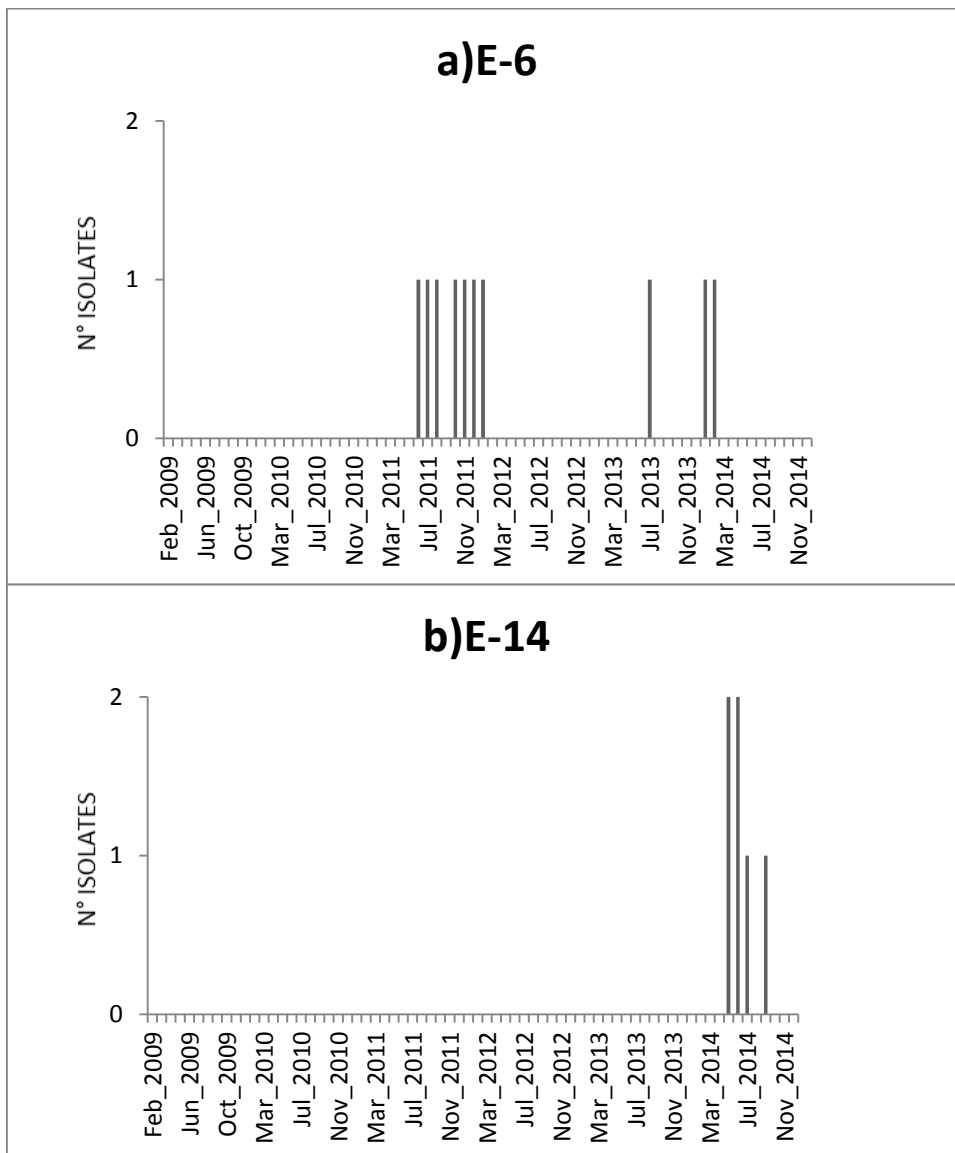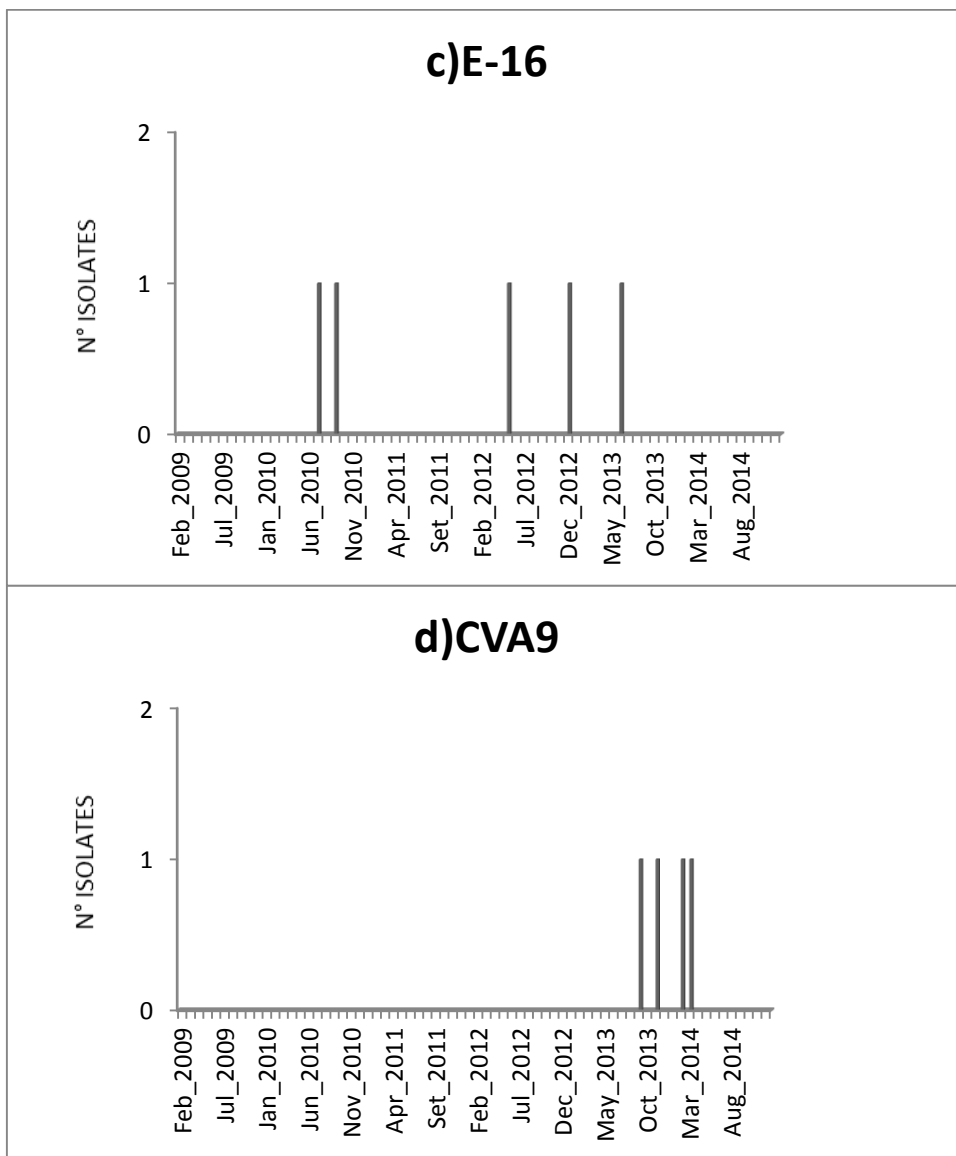
Figure 3: Seasonal patterns of six predominant circulating EVs in Cordoba city from 2009 to 2014. Monthly distributions of E-6, E-14, E-16, CVA9, CVA24, and E-20 are illustrated in panels a to f, respectively.
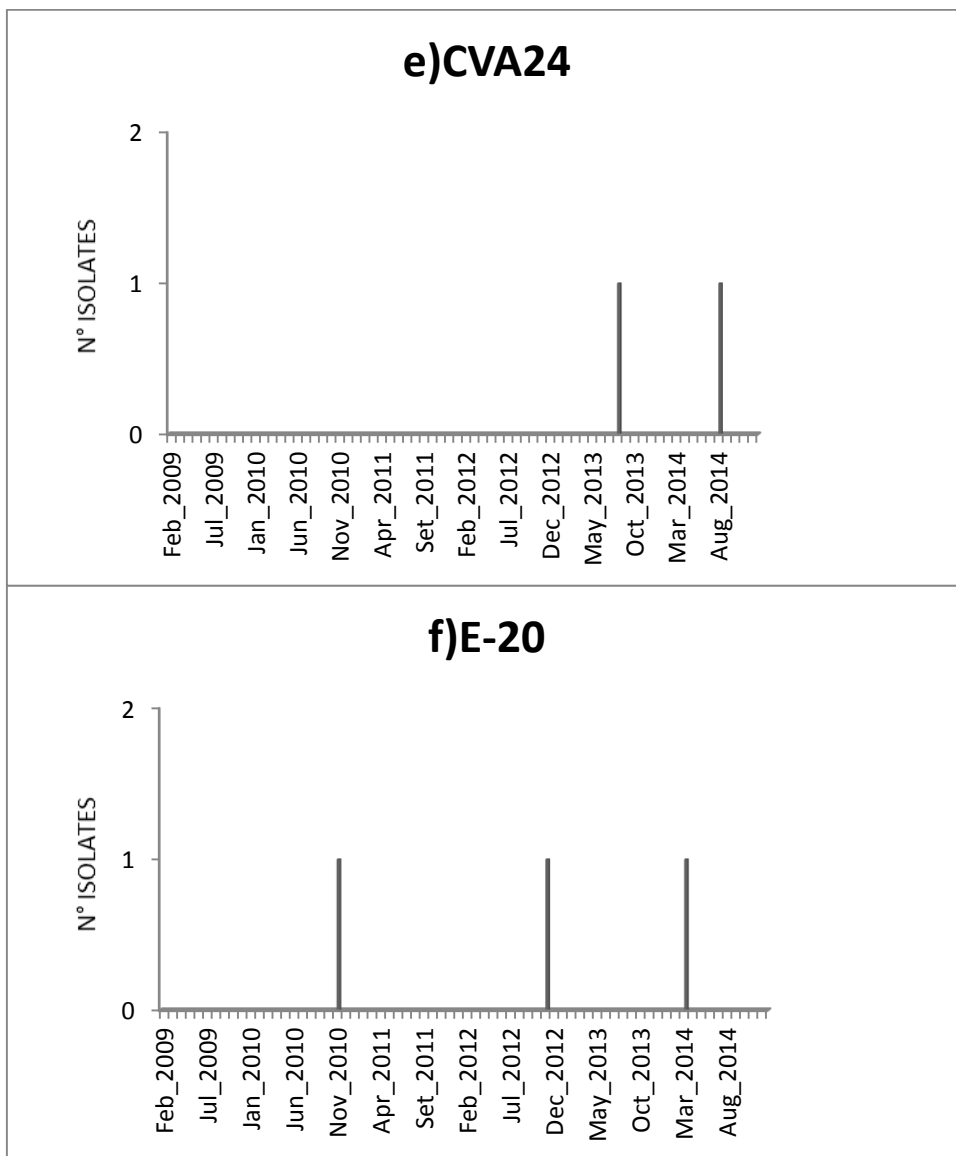
c)E-16

d)CVA9

e)CVA24

f)E-20

Table 1: NPEV serotypes detected in the sewage of Cordoba City, each year from 2009 to 2014.

Serotype	N° detected						
	2009	2010	2011	2012	2013	2014	Total(%)
E-6	0	0	6	1	1	2	10(28)
E-14	0	0	0	0	0	6	6(17)
E-16	0	2	0	2	1	0	5(14)
CVA9	0	0	0	0	2	2	4(11)
E-20	0	1	0	1	0	1	3(9)
CVA24	0	0	0	0	1	1	2(6)
Others(n)	1(E-7)	1(CVB4)	1(E-21)	0	1(E-25)	1(E-13)	5(14)
Total	1	4	7	4	6	13	35 (100)