

RESEARCH LETTER – Pathogens & Pathogenicity

Coxsackievirus B4 sewage-isolate induces pancreatitis after oral infection of mice

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One sentence summary: Sewage virus induced pancreatitis *in vivo* but clinical samples did not.

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ABSTRACT

Numerous serotypes which belong to the genus *Enterovirus* (EV) show variability in their virulence and clinical manifestations. They are also known to undergo changes caused by mutations and recombination during their circulation in the environment and the population. Various EV serotypes are prevalent in groundwater, wastewater and surface waters. Our previous studies showed that oral infection induces pancreatitis depending on specific conditions, such as gravidity, in an outbred murine model. Our aim in the present study was to further explore the pancreatic histopathology in an outbred mouse model following oral infection with clinical isolates from a patient who had aseptic meningitis and an isolate from a treated-sewage sample recovered from the residential area of the patient. The isolates were identified as coxsackievirus B4 (CVB4) in tissue culture. The CVB4 sewage-isolate induced pancreatitis after oral infection. In contrast, pancreatitis was absent following infection with the clinical isolates. Comparison of polyprotein sequences showed that the treated-sewage strains differed from the patient's isolates by 9 and 11 amino acids. We conclude that the isolates of clinical and environmental origin differed in their pathogenic properties and showed genetic variation.

Keywords: coxsackievirus B4; pancreatitis; isolates; pathogenesis; genetic variability

INTRODUCTION

Coxsackie B viruses (CVB) belong to the *Enterovirus B* species of the genus *Enterovirus* (EV), one of the 158 species of the Picornaviridae family <https://www.picornaviridae.com/>. Typical

spread of coxsackieviruses (CVs) is by the fecal–oral route, but they are also known to spread via aerosols (Pallansch and Ross 2006). Excretion of virus particles and their ability to survive in different environmental conditions make their circulation in

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the ground and in sewage waters presumable. CVs are known for their cardio- and pancreo- tropism and have been associated with autoimmune diseases such as chronic myocarditis and type 1 diabetes, as reviewed by different authors (Chapman and Kim 2008; Hober and Sane 2010; Precechtelova et al. 2014a).

CVs are susceptible to genetic changes (mutations and recombination) similar to other EVs, resulting in genetic diversity (Li et al. 2005; Hu et al. 2011). Their virus-encoded RNA polymerases are prone to errors therefore causing high mutation rates and form a swarm of virus particles of a genetically related viral cloud known as 'quasispecies' (Vignuzzi et al. 2006; Domingo, Sheldon and Perales 2012; Muslin et al. 2019). The amount of mutations is critical for adaptation, spread and pathogenesis of the virus (Pfeiffer and Kirkegaard 2005; Vignuzzi et al. 2006; Lauring and Andino 2010; Muslin et al. 2019). Similarly, RNA recombination also increases the adaptability of EVs to the environmental pressures (therapeutic and immune pressures) in the host (Oprisan et al. 2002; Lindberg et al. 2003; Oberste, Maher and Pallansch 2004; Simmonds and Welch 2005).

In our preliminary work (Sojka 2012), we observed differences in the pathogenicity of CV isolates from different origins. Based on that study and our other previous findings (Bopegamage et al. 2005; Precechtelova et al. 2014b) the aim of the present work was to compare histopathology of pancreatic tissue from mice orally infected with different isolates from: (1) stool and (2) the cerebrospinal fluid (CSF) of an aseptic meningitis patient and (3) treated sewage (recovered from the municipal plant allocated to the residential area of the patient). We then confirmed the histopathological findings with a double-blind experiment, which led us to examine genetic differences and similarities of the selected isolates. (Fig. 1 shows the experimental design).

MATERIALS AND METHODS

Cells

The following cell lines were obtained from the Public Health Authority in Bratislava, Slovak Republic: GMK (green monkey kidney) for the propagation of CVB4 isolates; RD (rhabdomyosarcoma) used in the laboratory isolations; and VERO (African green monkey kidney) and GMK for the virus passages and plaque purifications. Cells were grown in Eagle's minimum essential medium (MEM (E)) (Lonza, Basel Switzerland) supplemented with 10% heat-inactivated bovine serum (at 56°C) for cell growth and 2 or 5% serum for infection or maintenance of cells. Antibiotics were added to the culture media: 1000 U/mL penicillin and 0.01 g/mL streptomycin. The cells were incubated at 37°C.

Virus isolates

The isolates used in this study were from the Public Health Authority in Bratislava within the framework of the enterovirus surveillance program of the Slovak Republic (Klement et al. 2013), where they were isolated and identified as coxsackievirus (CVB4) in tissue culture (we are grateful to Z. Sobotova and K. Pastuchova).

Details of the isolates from a seriously ill aseptic meningitis patient:

CVB4 AS isolates from a single stool sample, identified as CVB4, isolated and passaged three times in RD cells: isolate numbers {1} 1 passage in GMK cells and {1a} 2 passages in GMK cells;

CVB4 AL isolates from the CSF of the same patient identified as CVB4, isolated and passaged three times in RD cells: isolate

numbers {2} 1 passage in GMK cells and {2a} 2 passages in GMK cells.

Details of the treated-sewage isolates:

CVB4 COV isolate from a sample collected from the tank of a municipal sewage plant from the residential area of the patient, were identified as CVB4: isolate numbers {3} three times passaged in RD cells and twice in GMK cells; plaque purified isolate numbers denoted as {3a} 1 passage after plaque purification on VERO cells and {3b} seven passages after plaque purification on VERO cells.

Human ethical committee approval was not required.

Mice infections

Outbred CD1 male mice (Envigo++++, Udine, Italy) were used. In the first study, (i) 3–4-weeks-old mice (10–15 g) and the second set which was a smaller experiment (ii) 7–8-weeks-old (18–25 g) were used. In set (i), all isolates were used for infection: CVB4 AS, CVB4 AL and CVB4 COV. In set (ii) the results from set (i) were confirmed in a double-blind experiment using only the CVB4 COV isolate {3b}.

^{4,5} Mice were infected as described previously by our group (Bopegamage et al. 2003; Bopegamage et al. 2005). All mice were orally infected with a dose of 0.2 mL of 10 TCID₅₀/0.1 mL, (tissue culture infectious dose) with 5 mice per interval and per isolate. Each set of experiments included a control group of mice also with 5 mice/group/interval. The control group (mock infected) was given 0.2 mL of phosphate buffered saline (PBS) orally.

Mice were housed three or two per cage in sterile pathogen free (SPF) conditions, supplied with sterile water and commercial food pellets (Top Dovo, Brno, Czech Republic). In infection set (i), blood and hearts, as well as pancreases, spleens and brains of the infected and control mice were collected at 0, 3, 5, 10 and 45 days post-infection (p.i.). In infection set (ii) the same organs were collected but we focused only on day 0 and on 3 and 5 days p.i. For histopathological analyses, organs were fixed in 4% formaldehyde solution (Bopegamage et al. 2003, 2005). This study was conducted with the approval of the Ethical Committee of the Slovak Medical University and the State Veterinary and Food Control Authority of the Slovak Republic: Numbers: C.k Ro 3035/07–221/3 and C.k. Ro 3248/16–221.

Histopathology

Histopathological changes were determined by hematoxylin eosin (HE) staining. Serial 4–7 μm thick sections of formalin fixed paraffin embedded organs were stained (pancreas, heart, spleen and brain) and examined for evidence for inflammatory as well as morphological and necrotic changes as described previously (Bopegamage et al. 2003, 2005). Presently we have compared only the HE staining of the pancreatic tissue from set (i) and set (ii). Infiltration and necrosis in the tissue were graded as described by Opavsky et al. (1999). A score of '0' absence of inflammation, infiltration or necrosis, '1' incipient, focal inflammation or necrosis (only one or two foci in the entire section), '2' mild to moderate infiltration or necrosis (10–40% of section affected), '3' moderate infiltration (40–70% of section affected), '4' extensive areas of infiltration or necrosis (70–100% of section affected).

RNA Extraction, reverse transcriptase (RT)-PCR and quantitative real-time (qRT) -PCR

Viral RNA was extracted from the viral isolates (clinical samples and treated sewage sample) after plaque purification, using the PureLink RNA Mini Kit (Invitrogen and ThermoFisher Scientific,

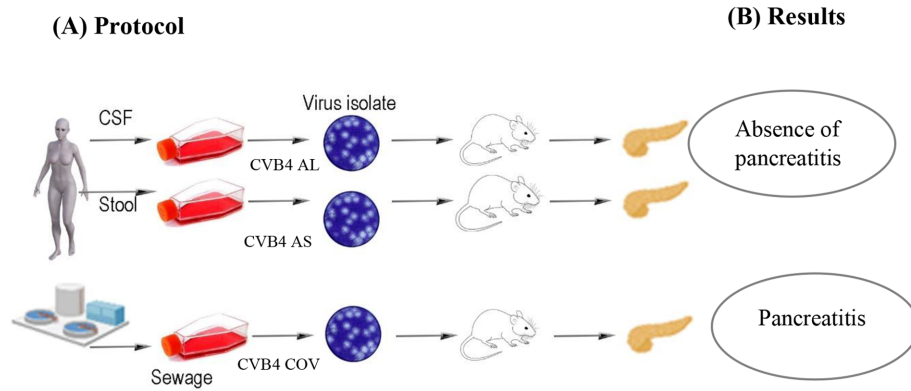


Figure 1. Schematic diagram of (A) main experimental protocol (infection of 3–4-week-old outbred CD1 male mice) and (B) results. CVB4, coxsackievirus B4; CSF, cerebrospinal fluid. This figure created with Biorender.com.

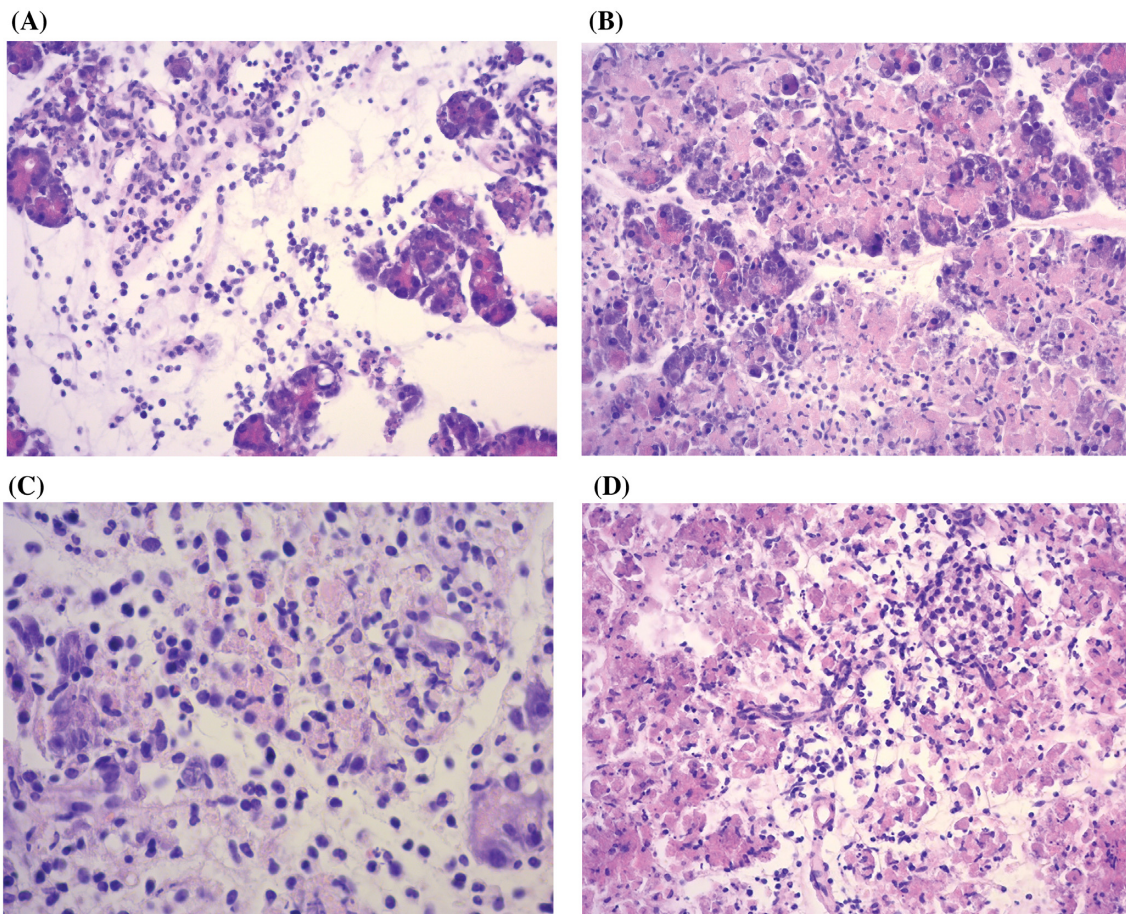


Figure 2. Sections of pancreatic tissues from 7–8-week-old outbred CD1 male mice infected with treated-sewage isolate CVB4 COV {3b} in infection set (ii) stained with hematoxylin and eosin. (A) Day 3 p.i., interstitial infiltrate, magnification 20x; (B) Day 3 p.i., acute pancreatitis, magnification 20x; (C) Day 5 p.i., acute pancreatitis, magnification 40x and (D) Day 5 p.i., acute pancreatitis, magnification 20x. CVB4: coxsackievirus B4; p.i.: post-infection.

Waltham, MA) as per the manufacturer's protocol. RNA isolates were stored at -80°C for further analysis. cDNA synthesis and cDNA amplification were performed by using a single tube method with the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) and nested reaction, as described before using Platinum PCR Super Mix (Invitrogen and ThermoFisher Scientific). Primers for the non-coding region of the EV genome for PCR reactions were obtained from Microsynth (Microsynth Seqlab GmbH, Göttingen, Germany)

as described previously (de Leeuw et al. 1994; Precechtelova et al. 2014b). Horizontal agarose gel electrophoresis (Subcell GT Agarose Gel Electrophoresis System, Bio-Rad, Hercules, CA) was used for detection and visualization of the PCR products. For visualization and analysis of DNA bands, Quantity One software (Bio-Rad) was used. The qRT-PCR reaction was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) thermocycler, using RealStar® Enterovirus RT-PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany).

Table 1. Nucleotide (A) and protein (B) alignment distance matrixes illustrating differences in the numbers of nucleotides and amino acids among different enterovirus sequences [between the samples].**(A) Nucleotide alignment distance matrix**

Origin of the sample	Number of passages after 3 passages in RD cells	Sample labeling used in the text	CVB4 AL {2}	CVB4 AL {2a}	CVB4 AS {1}	CVB4 AS {1a}	CVB4 COV {3}	CVB4 COV {3a}
Human CVB4 (from CSF)	1 passage in GMK	CVB4 AL {2}		0	3	8	84	97
Human CVB4 (from CSF)	2 passages GMK	CVB4 AL {2a}			3	8	84	97
Human CVB4 (from stool)	1 passage in GMK	CVB4 AS {1}				6	83	96
Human CVB4 (from stool)	2 passages GMK	CVB4 AS {1a}					88	101
Environmental CVB4 (sewage)	2 passages GMK, 1 passage after plaque purification on VERO cells	CVB4 COV {3}						34
Environmental CVB4 (sewage)	2 passages in GMK, 7 passages after plaque purification on VERO cells	CVB4 COV {3a}						

Sequences in the rows are compared with sequences in columns, all combinations are depicted. Sequences used for comparison were 7278 nucleotides long. The first two columns explain the origin of the sample and method of isolation for the isolates. The red frame contains only the clinical samples—original and their isolates. Both of the clinical samples (from CSF and stool) isolates have similar passage histories. The greatest variation in nucleotides was found for the original stool sample and its isolate (six nucleotides) while only three nucleotide differences were found for the original CSF sample and its isolate. The green frame contains only the treated-sewage sample and its isolate. A total of seven passages were performed on the treated-sewage sample and there were 34 nucleotide differences found between the sample and its isolates but only two different amino acids. Considerably higher numbers of nucleotide variations were found among clinical samples and treated-sewage samples (rightmost columns). CSF, cerebrospinal fluid; GMK, green monkey kidney; RD, rhabdomyosarcoma.

Next Generation Sequencing (NGS) and phylogenetic analysis

NGS sequencing protocol was performed according to Cinek *et al.* (2019). Briefly, viral RNA from samples (from clinical and treated-sewage isolates) was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD) on a QiaCube instrument. Reverse transcription was done using random hexamer primers and ImProm-II Reverse Transcription System (Promega, Madison, WI). The fragments were denatured and second strand synthesis was performed. Samples were purified by AMPure XP magnetic beads (Beckman Coulter Genomics). Libraries were then constructed using the Nextera XT kit (Illumina, San Diego, CA) and sequenced on a MiSeq instrument (Illumina).

Sequence analyses were performed using the Geneious software (Version 8.1.6 and 7.1.9, Auckland, New Zealand). Phylogenetic distance of found genomes is based on the alignment of the whole nucleotide sequences and alignment of the whole polyprotein sequences. The CVB4 genomes were uploaded into the GenBank database (National Center for Biotechnology Information [NCBI]; Bethesda, MD) and given accession numbers (please see results section, below).

RESULTS

Histopathological changes in the pancreas

Infection set (i) of 3–4-weeks-old mice (main experiment protocol) showed histopathological changes in the pancreas, with

Table 1. Continued.

(B): Protein alignment distance matrix

Origin of the sample	Number of passages	Sample labeling used in the text	CVB4 AL {2}	CVB4 AL {2a}	CVB4 AS {1}	CVB4 AS {1a}	CVB4 COV {3}	CVB4 COV {3a}
Human CVB4 (from CSF)	1 passage in GMK	CVB4 AL {2}		0	0	0	9	11
Human CVB4 (from CSF)	2 passages GMK	CVB4 AL {2a}			0	0	9	11
Human CVB4 (from stool)	1 passage in GMK	CVB4 AS {1}				0	9	11
Human CVB4 (from stool)	2 passages GMK	CVB4 AS {1a}					9	11
Environmental CVB4 (sewage)	2 passages GMK, 1 passage after plaque purification on VERO cells	CVB4 COV {3}						2
Environmental CVB4 (sewage)	2 passages in GMK, 7 passages after plaque purification on VERO cells	CVB4 COV {3a}						

Sequences in the rows are compared with sequences in columns, all combinations are depicted. Sequences used for comparison were 2184 amino acids long. The first two columns explain the origin of the sample and method of isolation for the isolates. The red frame contains only the clinical samples—original and their isolates. Both of the clinical samples (from CSF and stool) isolates have similar passage histories. No differences in amino acids were found after translation into protein sequences. The green frame contains only the sewage sample and its isolate. A total of seven passages were performed on the treated-sewage sample. Differences in only two different amino acids was observed. Considerably higher numbers of amino acid variations were found between clinical samples and treated-sewage samples (rightmost columns). CSF, cerebrospinal fluid; GMK, green monkey kidney.

acute pancreatitis observed only in mice orally infected with CVB4 isolates from treated sewage (Fig. 1). These findings led us to conduct infection set (ii), a double-blind experiment.

In infection set of the set (ii), in pancreatic tissue of 7–8-week-old orally infected mice, we confirmed the results from infection set (i), despite the different age of the mice. The pancreases of mice infected with the CVB4 COV isolate from treated-sewage showed infiltration of the exocrine pancreas (interstitial infiltrate; 4/5 mice) of grades 2 and 3, and acute pancreatitis of grade level 3 (1/5 mice) was observed at day 3 p.i. (Fig. 2A and B). Acute pancreatitis was present in all 5 mice at day 5 p.i. (Fig. 2C and D). Infiltration was absent in the pancreatic islets. In the control group of mock-infected mice pathological changes were absent in both endocrine and exocrine pancreas and infiltration was absent in pancreatic islets.

Whole genome sequencing of the isolates

We compared the clinical samples (CSF and stool) that included the original samples and their isolates. The results are summarized in Tables 1A and B. The red frame in Table 1A shows that more nucleotide differences were found between the original stool sample and the passaged isolate (six nucleotide differences). Whereas differences in three nucleotides were seen between the original CSF sample and its passaged isolate. The treated-sewage sample and the passaged isolate (in Table 1A green frame) differed in 34 nucleotides, which may reflect higher number of passages. The differences in amino acids are summarized in Table 1B. No differences were found among the clinical samples (Table 1B, red frame) and only two changes were seen between the treated-sewage sample and the passaged isolate

(Table 1B, green frame). Considerably higher numbers of differences were found between the clinical and treated-sewage samples.

The closest known sequence to our samples is that of accession number KY369904 (CVB4 strain EPV3715) and the similarity to it is 86%. Accession numbers of the sequence data for our samples at the GenBank NCBI are MW390784, MW390785, MW390786, MW390787, MW390788 and MW390789.

DISCUSSION

Mice are not the natural hosts of human EVs, yet they are good models for studying the pathogenesis of CVs (Bopegamage *et al.* 2020). In the present study, we demonstrated differences between clinical isolates collected from a seriously ill aseptic meningitis patient, and an isolate from a treated-sewage sample recovered from a regional municipal sewage treatment plant allocated to the residential area of the patient. A striking difference was observed between the histopathology of pancreatic tissues from outbred CD1 mice infected with the clinical versus the treated-sewage isolates. Only the two sewage isolates induced pancreatitis after oral infection; age of the mice was irrelevant. This finding was unique, as our previous studies showed absence of histopathological changes in outbred immunocompetent mice such as Swiss albino and CD1 mice (Bopegamage *et al.* 2005; Precechtelova *et al.* 2014b). We had observed pancreatitis only after oral infection of gravid dams and their challenged pups (Bopegamage *et al.* 2012; Sarmirova *et al.* 2019), only by the CVB4 E2 strain.

Our previous experience (Al-Hello *et al.* 2008) showed that an isolate from a newly diagnosed diabetic child and identified as coxsackievirus A9 (CAV-9) was capable of infecting mouse pancreatic islet cells *in vitro* (Bopegamage and Petrovicova 1994). This isolate also caused cytolysis and functional damage of primary cultures of human pancreatic islets (Al-Hello *et al.* 2008). Full-length genome sequencing and molecular characterization showed that the isolate was echovirus 11 (E-11). Phylogenetic analyses demonstrated that E-11/D207 was closely related to a specific subgroup B of E-11 strains known to cause uveitis. The isolate showed antigenic regions of capsid proteins of E-11 and cross-reacting with CAV antiserum. Thus, in the present study we were interested in comparing the treated-sewage sample and clinical isolates at the genetic level.

Whole genome sequencing documented the fact that extent of genetic changes were associated with repeated virus passaging, versus the a priori distance between the currently obtained clinical and treated-sewage isolates. Although some changes were induced over the course of passages, the baseline difference between isolates of the same serotype was still apparent.

The clinical and treated-sewage isolates were identified as CVB4 by both tissue culture and NGS. We did not find evidence of coinfection in clinical specimens, but individual sequence comparisons and phylogenetic analyses, showed 86% agreement with the nearest virus detection. The number of cell culture passages or plaque purification affected the genomic sequences especially for the treated-sewage isolates to some extent, which differed by seven passages.

Evolutionary changes in the EV genome, including recombination, insertions and substitutions, contribute to genetic diversity and affect virus replication, pathogenicity and fitness (Kawamura *et al.* 1989; Zell *et al.* 1995; Gromeier *et al.* 1999; Chapman *et al.* 2000; Harvala *et al.* 2002; Jahan, Wimmer and Mueller 2011). Endemic or sporadic EVs undergo changes through mutations and recombination during their circulation in the host and

environment resulting in altered or increased viral pathogenicity (Muslin *et al.* 2019). Deletion in the 5' terminal region of CVB has been shown to affect viral persistence in the pancreas of experimentally infected mice (Tracy *et al.* 2015). A single mutation in the VP1 region of EV-A71 increased virulence and neurotropism *in vivo* (Caine *et al.* 2016). Our findings show that not all nucleotide changes appeared as amino acid sequence changes, thus reflecting the redundancy of the genetic code and also that only viable variations can remain stable. Our studies also raise several questions that should be further investigated such as: (1) are genetic differences between the treated-sewage and clinical samples related to differences in their pathogenicity in mice? (2) do these differences help the virus to infect or adapt to the living being such as mouse or human? and (3) are the genetic changes related to the virus tropism?

A limitation to our study is that we did not have a sewage isolate directly from the sewage tank of the patient's house, although we had the stool sample. The treated-sewage sample came from tank of the municipal sewage the treatment plant allocated to the residential area of the patient, so it is possible that the isolate may be another variant circulating in the area. However, this likelihood should be low as there were only three isolates reported in this area that particular year (CVB4, CVB5 and ECHO13; Klement *et al.* 2013).

From our findings, we may conclude that isolates of clinical and environmental origin, although both identified as similar serotypes, differed in their tropism and virulence in mice which may in turn be related to the differences in genomic sequences.

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Conflicts of interests. None declared.

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