Survival for the identification of cases of acute respiratory infection by enterovirus D68 in children in a tertiary level care hospital during 2014-2016


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Abstract

Background: The reemergence of enterovirus (EV) D68 infections in the United States was reported from August-October 2014 (691 cases). In Mexico, an outbreak at the National Institute of Respiratory Diseases was reported (24 cases). The results of epidemiological surveillance (ES) of enterovirus and other respiratory viruses in a national pediatric tertiary care hospital are presented.

Methods: Following the alert issued by the reemergence of EV-D68 in 2014, ES—which only detected respiratory viruses by PCR in patients with influenza-like illness using nasopharyngeal swabs—expanded to include children with asthma exacerbation or acute respiratory distress. Positive samples for Enterovirus sp. (EV) were confirmed and typed by sequencing. Subsequent sequencing was used to obtain the complete viral genome.

Results: Of 1705 samples, 13 were positive to EV. Patients with EV presented the following comorbidities: chronic lung disease (7.7%), neoplastic disease (15.4%), allergic asthma/rhinitis (23%), recurrent pneumonia (23%), and other (23%). Of the 13 samples positive for EV, three were positive for EV-D68. These cases required invasive mechanical ventilation, presented no neurological involvement and survived.

Conclusions: The impact of the population studied by EV-D68 was lower than that reported in the country during the same period. Cases of EV-D68 infection had multiple comorbidities, but few pulmonary comorbidities, which could explain the low attack rate. The ES and infection prevention system may have contained the outbreak.

Key words: Human enterovirus D. Pneumonia. Epidemiology. Pediatrics.
The human enterovirus D68 (EV-D68) is a member of the enterovirus D species belonging to the genus Enterovirus and the Picornaviridae family. Only five serotypes have been identified. In 1962, rhinovirus type 87 (HRV87) was detected in a hospitalized pediatric patient with respiratory infection in California, USA. Recently, HRV87 was reclassified as EV-D68, considering the phylogenetic and cross-serological neutralization analyzes. Strains of EV-D68 are classified into three lineages based on the sequence of the VP1 gene. The strains of lineages 1 and 2 form groups in the same geographical region while strains of lineage 3 do not have a specific geographical pattern of circulation. These lineages, described by Meijer et al., correspond to groups B, C and A, respectively, and were characterized by Tokarz et al. Recently, group D was described. The clinical spectrum included acute respiratory infections with varying degrees of severity, from an acute respiratory disease of the upper respiratory tract to severe pneumonia; this last condition is the most frequent.

In the Americas, several cases of EV-D68 have been reported: 17 cases in Canada in 2014 and 26 cases in the United States from 1970 to 2005. From August to October 2014, an increase was reported, with a total of 691 cases in 46 states of the USA; the majority occurred in children with severe respiratory disease, producing fatal cases. In Mexico, an outbreak of 24 cases of infection by EV-D68 was reported in hospitalized children with pneumonia or exacerbation of asthma, which corresponded to 19% of cases with acute respiratory infection, with cough and dyspnea as predominant symptoms. Also, the infection was associated with lymphopenia and propensity to develop hypoxemia. Due to the increase in cases of EV-D68 worldwide, it is considered as a re-emerging pathogen.

The Hospital Infantil de México Federico Gómez (HIMFG) is a tertiary national referral hospital. It receives around 7000 patients per year from all the states of the country. Approximately 80% of the attended patients present some degree of immunocompromise, mainly due to different types of cancer.

This article shows the result of the epidemiological surveillance during the reemergence and alert for EV-D68 (October 2014-January 2016) in the HIMFG, as well as the molecular characterization of the viral strain. Also, other respiratory viruses identified during this period are shown. These data were obtained from the protocol with registry HIM/2012/031, approved by the Research Committee of the HIMFG.

**Introduction**

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**Methods**

**Epidemiological surveillance**

Since January 2013, systematic and routine surveillance for the detection of respiratory viruses has been carried out in HIMFG in all patients with data on severe acute respiratory infection (SARI) and influenza-like illness (ILI), both of nosocomial and community origin. Inclusion criteria considered for ILI are fever or history of fever greater than or equal to 38° C, cough and headache, accompanied by one or more of the following signs or symptoms: rhinorrhea, coryza, arthralgias, myalgias, prostration, odynophagia, chest pain, abdominal pain, nasal congestion or diarrhea. In children under five years, irritability replacing headache is considered a cardinal sign. In patients older than 65 years or immunocompromised patients, fever is not considered as a cardinal sign.

The inclusion criteria considered for SARI are breathing difficulty, history of fever greater than or equal to 38° C and cough, with one or more of the following symptoms:
Detection of respiratory viruses by low-density microarrays

The diagnosis of the respiratory viruses was made using low-density microarrays, operating the CLART® PneumoVir platform (GENOMICA), following the manufacturer’s instructions for the detection of the following viruses: adenovirus; bocavirus; coronavirus; enterovirus (echovirus); influenza virus A (subtypes human H3N2, human H1N1 and H1N1/2009), B and C; metapneumovirus (subtypes A and B); parainfluenza virus 1, 2, 3 and 4 (subtypes A and B); rhinovirus; respiratory syncytial virus type A (RSV-A); respiratory syncytial virus type B (RSV-B).

Specific identification and obtaining of the genome of the EV-D68

Because enterovirus infection can be confirmed by cell culture or specific PCR, viral RNA was extracted from the positive samples using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer’s recommendations. The enterovirus/rhinovirus differentiation by semi-nested RT-PCR with primers EV1, EV2 and EV3, and the subsequent specific identification of EV-D68 by real-time RT-PCR were carried out following the previously described protocols. Once the positive samples were identified to EV-D68, sample 2351 obtained from case 3 was selected, because it presented a CT value (cycle threshold) of 26.5 (lower than those of samples from cases 1 and 2) in the real-time RT-PCR diagnostic test. This value indicates a suitable viral load value for the amplification of the genomic fragments through the technique of sequencing of amplification products, using massive parallel sequencing.

For this purpose, PCR primers were designed for the total coverage of the genome using three semi-nested RT-PCR reactions (Table 1), using the Primer 3 Plus program and as a target, the genomic sequence of strain US/MO/14-18947, with accession number KM851225 of GenBank. The SuperScript® III Platinum® One-Step qRT-PCR kit (Invitrogen) was used, with the reagent concentrations recommended by the manufacturer and the initiators at a concentration of 30 pmol per reaction. The conditions for both the first and the second round of amplification were 30 minutes at 50° C for retrotranscription, 2 minutes at 95° C for the activation of Taq polymerase, followed by 40 cycles of 15 seconds at 95° C for denaturation, 30 seconds at 53° C for the alignment of the primers and 4 minutes at 72° C for the extension, in addition to a final extension step of 72° C for 5 minutes. Once the amplification products were generated, a mixture of them was made at a final concentration of 2 ng/μl, and the libraries were generated for sequencing using the Nextera XT kit, according to the manufacturer’s instructions. Sequencing was performed on the MiSeq® (Illumina) using 150 cycles. At the end of the sequencing, mapping the sequence with the software Newbler v. 2.6 made the assembly, using the sequence of HEVD68ref-NY329 (accession number KP745767.1) as a reference. The average depth obtained was 1300X.

Phylogenetic analysis

We performed the analysis of 98 genomes of the EV-D68 available in the GenBank database and those obtained from different countries isolates where outbreaks have occurred, using the MEGA6 software, with the maximum likelihood method based on the Tamura-Nei substitution model and Gamma distribution.

Results

From October 2014 to January 2016, 1705 nasopharyngeal samples were taken with a percentage of positivity for any respiratory virus of 49.85% (N=850) (Table 2), with an interquartile age range of 2 to 8 years old and a median of 4 years old; 360 patients (42.4%) were female. Rhinovirus was the most prevalent virus, with 28.1% (239 cases), followed by the respiratory syncytial virus (RSV) with 23.6% (201 cases) and co-infections in 17.3% (147 cases). The percentage of positive samples for Enterovirus sp. it was 1.5% (n=13). Of the positive samples to enteroviruses, 53.8% (n=7) were cases of co-infection: two cases of co-infection with rhinovirus and two cases with three identified viruses. The age of patients infected with enterovirus was between 2 and 7 years old (mean 3.1 years and median 5 years). Four patients (30.7%) were female and had the following comorbidities: one with chronic lung disease (7.7%), two with neoplastic disease (15.4%), three with asthma/allergic rhinitis (23%), three recurrent pneumonias (23%), three with other pathologies (23%) and one healthy patient (7.7%). Three cases were detected with positive PCR to EV-D68 (4.3% of cases positive for enteroviruses), which
did not present a neurological affection. The cases are described as follows:

**Case 1**

A male infant of one year and nine months of age with acute malnutrition, history of wheezing and two previous hospitalizations for community-acquired pneumonia was presented. Asthmatic mother. Immunizations according to national vaccination scheme (except flu vaccine). He was admitted to the emergency room due to irritability and respiratory distress, without documented fever. Physical examination revealed bronchospasm, 76% O₂ saturation and decompensated respiratory acidosis; required mechanical ventilation for 72 hours. The results of his laboratory tests were hemoglobin (Hb) 12.7 g/dl, hematocrit (Hct) 38%, leukocytes 8,100/µl, neutrophils 80.2%, lymphocytes 13.6%, platelets 266,000/µl. The patient received antibiotic treatment with cefotaxime, dicloxacillin and clarithromycin due to severe pneumonia. On the day of admission, a nasopharyngeal swab was performed, identifying enteroviruses. Subsequently, partial sequencing and real-time RT-PCR confirmed EV-D68.

The endotracheal suctioning sample showed normal biota development and blood cultures showed no bacterial development. Bilateral diffuse interstitial infiltrate was observed on the chest radiograph (Fig. 1). On the sixth day of hospitalization, she was discharged because of his improvement.

**Case 2**

Male infant of 1 year and 7 months old, with a history of severe community-acquired pneumonia and supraventricular tachycardia at 9 months. He was evaluated by the Cardiology service without requiring pharmacological management. He went to the emergency room due to a runny nose and cough, without documented fever. Physical examination revealed progressive respiratory distress, 67% O₂ saturation, hypotension (requiring advanced resuscitation maneuvers), fluid therapy, and mechanical ventilation. The laboratory results were the following: Hb 9.6 g/dl, Hct 29.4%, 17,300 leukocytes/µl, neutrophils 80%, lymphocytes 16%, platelets 204,000/µl. Arterial blood gases showed metabolic acidosis (with lactate at 5.5 mmol/l). He received antibiotic treatment with ampicillin-sulbactam. On the day of admission, a nasopharyngeal swab was performed, identifying enterovirus. Subsequently, EV-D68 was confirmed by partial sequencing and
real-time RT-PCR. No co-infection with bacteria was identified (by blood cultures) neither other respiratory viruses were found. The chest radiograph showed data of air trapping, horizontalization of costal arches, the collapse of hemidiaphragms and increase of bronchopulmonary pattern (Fig. 2). The length of hospital stay was 7 days.

Case 3
A fifteen-year-old male (strain 2351) with a diagnosis of Hodgkin’s lymphoma, nodular sclerosis, stage IIB primary cervical-mediastinal, with early supra and infradiaphragmatic relapse in the second post-relapse cycle. He received the first cycle with ifosfamide, vinblastine, cytarabine and bretuximab; the second cycle, with vinorelbine, ifosfamide and cytarabine. After discharge, he came back because of cough and respiratory difficulty of one day of evolution. Physical examination revealed bilateral submandibular, mobile, non-painful adenopathies, thorax with decreased thoracic movements, low intercostal retraction, xiphoid retraction, hypoventilated pulmonary fields, basal wheezing and rales. Its temperature was 39°C, heart rate 159 beats/min, respiratory rate 19/min, oxygen saturation 88%, blood pressure 102/68 mmHg, weak pulses. Lab results were Hb 14.3 g/dl, Ht 42%, leukocytes 3500/μl, neutrophils 51%, total neutrophils 1785, lymphocytes 20%, monocytes 23%, eosinophils 5%, basophils 1%, platelets 210,000/μl, C-reactive protein 2.48 mg/dl, procalcitonin <0.10 ng/ml. Respiratory acidosis compensated with a lactate of 1.1 mmol/l. The chest radiograph showed a mixed diffuse bilateral infiltrate with no evidence of consolidation (Fig. 3). Upon admission, he had two charges with crystalloids at 20 ml/kg. The patient received treatment with cefepime (7 days) and amikacin (3 days), in addition to intravenous methylprednisolone and micronebulizations with salbutamol and ipratropium bromide. He required continuous positive airway pressure (17 hours). Subsequently, oxygen treatment with a facial mask with reservoir 10 l/min and then with nasal tips at 1 l/min. This allowed achieving a saturation higher than 90%. The length of hospital stay was 9 days. The diagnosis was community-acquired pneumonia by enterovirus. The patient was discharged with nasal tips at 1 l/min. The genome sequence of EV-D68 obtained was included in the GenBank database under the number of KT825142.

Discussion
The present research constitutes the second report on infantile cases of acute respiratory infection caused by enterovirus D68 in 2014 in Mexico. The National Institute of Respiratory Diseases (INER, for its Spanish acronym) reported the first 24 cases in a pediatric population of a center of respiratory diseases, with an average age of 5.2 years old and 54.2% of female patients. The mean age for the present cases was 6.1 years old, with exclusive involvement of male patients. The average age in both centers harmonizes.
In this report, patients presented initially cough, rhinorrhea and dyspnea, which matches with that reported in the INER\(^{10}\). The patients described were received in the emergency department with progressive respiratory weakening, hypoxemia and bronchospasm. In Spain, 58% of the cases with acute respiratory infection caused by enterovirus with hypoxemia were reported\(^{13}\). All three patients required admission to the intensive care unit. Infants did not present fever, but the high-grade fever was documented in the adolescent patient. In the INER, fever was reported in 67% of the patients\(^{10}\), in Kansas, USA, in 26% and Chicago, in 18%\(^{21}\). All the patients in this report required mechanical ventilation, unlike that published by Vázquez-Pérez et al., who reported that only 25% (n = 6)\(^{10}\) needed it. The patients did not present neurological affection, which agrees with the cases previously reported in Mexico\(^{10}\) and Spain\(^{13}\), unlike the USA reports, where a relationship of EVD-68 with flaccid paralysis was found, although it is a rare manifestation\(^{22}\). One of the cases in this report presented wheezing; in the INER, this symptom was present 75% of the patients\(^{10}\); in Kansas, in the 21%\(^{21}\) and Spain, recurrent wheeze was reported in 83%\(^{13}\). Significantly, one of the cases described in this study was identified with early childhood wheezing, an influential antecedent in cases of infection by EV-D68\(^{9}\). In the USA, a greater relationship with asthma of 68% and 73% was reported in Kansas and Chicago\(^{21}\), respectively, unlike that published in Mexico, which was only 37.5%\(^{10}\). Regarding the radiographic findings in this study, interstitial infiltrate and air trapping data were the mainly found; in Kansas parahilar infiltrate and atelectasis was reported\(^{21}\); INER reported 62.5% of the cases with opacities (pneumonia)\(^{10}\).

Although epidemiological surveillance was carried out in hospitalized patients to search for cases of EV-D68 in a reference hospital, only three cases were identified. This report does not agree with what was previously reported in Mexico, which includes a report of 24 cases in a single center in the same period. It is possible that the smaller number of cases identified in this work, in comparison with the report of the INER, is because the patients treated in the HIMFG are not predominantly asthmatic or with a history of bronchospasm, but rather immunocompromised patients with different type comorbidities.

The phylogenetic analysis showed that the strain MEX/DF/2014-InDRE2351 belongs to the B group of classification of EV-D68, which include the majority of the sequences obtained from cases of the USA outbreak in 2014, and it has been proposed that comes from the strain CA/AFP/11-176 (Fig. 4)\(^5\). The closest evolutionary relationship was established with the strain EV-D68/Homo sapiens/USA/SSSENT29/2014, identified in a pediatric patient (female 5 years old) in Massachusetts, USA, in September 2014. The Genome sequence of EV-D68 obtained from case 3 was included in the GenBank database with accession number KT825142.

In 2014, a moderate risk of transmission of EV-D68 was established based on the low circulation of the virus in the population, according to studies conducted in the European Union\(^{21}\). However, active surveillance should continue to detect new cases. For this reason, screening is recommended for the search for EV-D68 in negative cases to other respiratory viruses, in cases with an enterovirus/rhinovirus report and severe cases of acute respiratory infection\(^{21}\). Although in the HIMFG the epidemiological surveillance with nasopharyngeal swab tacked in cases with acute respiratory infection has been done for several years, and the bronchospasm was added due to the health alert, no more cases were found than those described or secondary transmission. The measures applied that could help prevent transmission were the strict application of contact and droplets isolation measures in all patients diagnosed with enterovirus, in addition to hand hygiene, which, during the study period, remained between 72-80%\(^{23}\).

This study suggests that it is possible that enterovirus D-68 does not frequently affect pediatric patients with significant comorbidities or immunocompromise, such as the usual patients of center. In contrast, patients with previous lung disease, as suggested by the Centers for Disease Control in Europe\(^{21}\) and in the Mexican paper that was previously reported earlier.
Figure 4. Phylogenetic tree based on complete genomes of EV-D68. The analysis included the strains from Mexico’s cases, the sequences obtained from the outbreak in USA during 2014 and representative strains of subtypes A, B, C and the newly described D. The sequences are marked by the accession number GenBank and designation of the strain.
from Vázquez-Pérez et al. they can be affected more frequently. In the surveillance study conducted in Europe, from July to December 2014 and with the participation of 13 countries, 4.1% of patients with immunocompromise were reported out of a total of 196 children less than 17 years of age with documented information. In the less than-2 group, they reported (with information available in 76% of the cases), chronic/recurrent respiratory problems in 21.9%, other comorbidities in 11%, immunocompromise in 3.3% and absence of comorbidity identified in the 63.7%.24

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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