Emerging Non-Polio Enteroviruses recognized in the framework of the Acute Flaccid Paralyses (AFP) surveillance system in Northern Italy, 2016–2018

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A B S T R A C T

Background: Acute Flaccid Paralyses Surveillance (AFPS) monitors the emergence of polioviruses and can track Non-Polio Enteroviruses (NPEVs). We report AFPS activity in the Lombardy region (Northern Italy) from 2016 to 2018.
Methods: Fecal and respiratory samples were collected from children <15 years who met the WHO definition of an AFP case, analyzed by virus isolation in cell cultures (RD/L20B) and by a one-step real-time RT-PCR assay specific for the 5′-noncoding-region of NPEV. NPEV-positive specimens were further analyzed by sequencing a fragment of the VP1 gene.
Results: 36 AFP cases (89 stool and 32 respiratory samples) were reported with an incidence of 1.1/100'000, 0.9/100'000, 0.6/100'000 children <15 years in 2016, 2017, 2018, respectively. Poliovirus was not identified, whereas NPEVs were detected in 19.4% (7/36) of AFP cases. The presence of one Echovirus-25 (2016), two EV- and D68 (2016 and 2018), one EV-A71 (2016), and one Echovirus-30 (2016) sharing high nucleotide identity with NPEVs detected in Europe was identified.
Conclusion: The absence of polio was confirmed. The unpredicted detection of emerging EV-D68, EV-A71, and E-30 sharing high sequence nucleotide similarity with viruses involved in the latest outbreaks, provided valuable and up-to-date information, emphasizing the importance of monitoring NPEVs through AFPS.

Introduction

The World Health Organization (WHO) strategic plan for global poliomyelitis (polio) eradication includes the implementation of infant immunization coverage by vaccination campaigns, national immunization days, and targeted "mop-up" campaigns, along with the active community-based PV investigation (WHO, 1996, 2021). Recently, Poliovirus (PV) transmission decreased considerably worldwide; however, Afghanistan and Pakistan are still reporting endemic cases, which pose a global risk of PV reintroduction.

Following WHO procedures, an acute flaccid paralysis (AFP) case is confirmed through the identification of all patients with polio-like symptoms in the framework of AFP Surveillance (AFPS) system and the subsequent analysis of biological samples to detect PV strains (i.e., wild or vaccine-derived), allowing a rapid investigation of confirmed cases and their community (WHO, 2019). AFPS is a sensitive case-based scheme that, concurrently with PV detection, can monitor the introduction and circulation of Non-Polio Enteroviruses (NPEVs) in a community (WHO, 2019).

With over 100 serotypes, NPEVs are a significant public health concern, especially since EV-D68, EV-A71, and Echovirus-30 (E-30) emerged as a cause of neurological complications – including AFP, acute flaccid myelitis (AFM), and brainstem encephalitis - in children (Suresh et al., 2018; Suresh et al., 2020).

Generally, EV-D68, EV-A71, and E-30 have been associated with clinical manifestations such as respiratory disease (EV-D68), hand,

foot, and mouth disease (HFMD, EV-71), and sporadic cases of aseptic encephalitis (E-30). However, since 2014, EV-D68 has also been reported as a cause of AFM epidemics (Messacar et al., 2018), EV-A71 caused an outbreak of brainstem encephalitis in 2017 (Casas-Alba et al., 2017), and an upsurge of E-30 has been observed across Europe since 2018, also associated with meningitis-related outbreaks with long-term sequelae (Broberg et al., 2018).

We analyzed the molecular epidemiology of NPEVs in AFP cases identified within the AFPS activity from 2016 to 2018 in Lombardy (Northern Italy) by molecular detection and characterization of NPEVs identified in respiratory and stool samples.

MATERIALS AND METHODS

This study was conducted from January 1, 2016, to December 31, 2018, in Lombardy, a region in northern Italy, accounting for nearly 1/6 of the national population with about ten million inhabitants. The Department of Biomedical Sciences for Health, University of Milan, which is one of the six Italian Sub-National Reference Laboratories (SNRLs), performing virological investigations on biological samples collected in the framework of the Italian AFPS, implemented at the national level since 1997 (Pellegrinelli et al., 2015). SNRLs strictly comply with WHO guidelines, methods, and recommendations for conducting AFPS (Pellegrinelli et al., 2015), are annually evaluated by the National Polio Reference Laboratory (NPRL) in the Department of Infectious Diseases of the Istituto Superiore di Sanità (ISS) for proficiency in isolation, detection, and typing of PV and NPEVs (Fiore et al., 1999). SNRLs share regional AFPS results with both the NPRL and the Italian Ministry of Health.

Acute Flaccid Paralysis Surveillance Systems

According to WHO case definitions, an AFP case is a child <15 years with a clinically evident AFP of one or more limbs with decreased or absent deep tendon reflex or presenting with a sudden onset of bulbar paralysis and person of any age with paralytic illness with suspicion of poliovirus infection (e.g., travel-associated). (https://www.who.int/immunization/monitoring_-surveillance/burden/vpd/surveillance_type/active/poliomyelitis_-standards/en/). AFP cases were notified by sending an "AFP case report," according to "zero reporting" methodology, to the reference SNRL twice a month. Prompt collection of (i) two stool specimens were taken 24–48 h apart within 14 days since the onset of paralysis, and, if available, (ii) a respiratory sample was taken within seven days since the onset of paralysis was requested for virological tests from each AFP case.

Epidemiological (type of PV vaccine received and date of administration), demographical (age, gender, country of origin), clinical symptoms (presence of fever at the onset, progression of paralysis, whether symmetric or asymmetric paralysis) and the date of onset, were collected in an 'ad hoc' questionnaire by physicians or healthcare workers (https://www.who.int/immunization/monitoring_surveillance/burden/vpd/surveillance_type/active/poliomyelitis_standards/en/).

Ethical statement

This study was performed according to the Institutional Review Board guidelines concerning the use of biological specimens for scientific purposes in compliance with Italian law (art.13 D.Lgs 196/2003). Patients' privacy and confidentiality issues were managed in complete agreement with national legislation. Approval from an ethics committee and informed consent for virus detection were not required since data and samples were collected and analyzed anonymously within the national AFPS system.

EVs identification and molecular characterization

Respiratory (n = 32) and stool (n = 89) samples collected from each AFP case were analyzed to detect NPEV and PV with viral isolation on cell cultures by using specific cells lines, following the WHO algorithm (https://www.who.int/immunization/monitoring_surveillance/burden/vpd/surveillance_type/active/poliomyelitis_standards/en/). In particular, RD cells (human rhabdomyosarcoma cells), permissive to all EVs, were used to determine the growth of any culturable EV, whereas L20B (murine transgenic L cells expressing the gene for the human cellular receptor for PV) were used to identify PV positive samples.

Contrary to respiratory samples, stool samples need to be processed before viral isolation, and the final suspension was prepared with 10% of stool samples in phosphate-buffered saline and clarified by micro-tube centrifugation (13,000 rpm for 15 min), as per WHO protocol (https://www.who.int/immunization/monitoring_surveillance/burden/vpd/surveillance_type/active/poliomyelitis_standards/en/).

For rapid virus identification, RNA was extracted from stool suspension, respiratory samples, and the lysate of cell cultures that showed a cytopathic effect (CPE), and a specific 5′ noncoding region (nucleotide [nt.] 179-575) of EVs was amplified by an in-house one-step real-time RT-PCR assay (Pellegrinelli et al., 2017a, 2017b).

The molecular characterization of EVs was performed by sequencing a fragment of the VP1/2A (nt. 2602-2977) gene (Nix et al., 2006). The nucleotide sequences were obtained by an automated DNA sequencing (Macrogen Inc, Korea); the study sequences were aligned with reference strains of previously published genotypes retrieved from a GenBank® database on the NCBI website (https://www.ncbi.nlm.nih.gov/genbank/) by using the ClustalW program implemented in the alignment editor BioEdit (Hall, 1999). Nucleotide sequence identity was calculated using the Sequence Identity Matrix tool of BioEdit software (Hall, 1999). The phylogenetic analysis was conducted by MEGA software, version 6.0 (Tamura et al., 2013).

Results

Demographical, epidemiological, and clinical characteristics of AFP cases

From January 1, 2016, to December 31, 2018, 36 AFP cases were reported in Lombardy for 89 stool samples and 32 respiratory samples collected for virological analysis.

The median age of AFP cases was 5.2 years (interquartile range [IQR]: 5.3 years), and 60.5% (23/36) of AFP cases were recorded in children <5 years old. Overall, 58.3% (21/36) of AFP cases were males. All children but one received inactivated polio vaccine (IPV) vaccination according to their age.

Nearly half (20/36; 55.6%) of AFP cases were identified by pediatric wards, followed by neuropsychiatric units (12/36; 33.3%) and intensive care units (4/36; 11.1%).

The vast majority of cases (83.3%; 30/36) presented with asymmetric paralysis, while fever over 38 °C was observed in 38.9% (14/36) of AFP cases and in 22.2% (8/36) of cases, paralysis progression occurred within four days from symptoms onset. Paralysis of all four limbs was observed in 25% of patients (9/36), while 13.8% of cases (5/36) had limb paralysis.

On follow-up forms, "not conclusive diagnosis" was reported in seven cases (7/36; 19.5%), five of them having an initial diagnosis of GBS and two cases of acute myelitis. Of the 29 clinical follow-ups (recorded 60-90 days from paralysis onset) with a conclusive diagnosis, 51.7% (15/29) reported GBS, 17.2 (5/29) were AFM. Acute myelitis and encephalitis, including meningoencephalitis and

brainstem encephalitis, were both identified in 10.3% (3/29) of AFP cases; also, hypotonia/asthenia or genetic/metabolic disorders were also reported in three AFP cases (10.3%).

Figure 1 shows the temporal distribution of AFP cases by month: a peak of cases was observed in November and December when 44.4% (16/36) of cases were notified (Figure 1).

Performance indicators of AFPS

According to the Lombardy's population size aged less than 15 years, 14 AFP cases per year were expected. During the study period, the annual AFP incidence rate was 1.1/100'000 children <15 years in 2016, 0.9/100'000 in 2017, and 0.6/100'000 in 2018. According to the WHO indicators, the sensitivity of AFPS fully met the criteria of the non-polio AFP incidence ($\geq$1.00/100'000) in 2016. Completeness of case investigation ($\geq$80%) was always achieved, being 93.3% (14/15) in 2016, 84.4% (11/13) in 2017, and 100% (8/8) in 2018. Completeness of clinical follow-up was achieved during this 3-year study since it was available in 80% (12/15), 84.6% (11/13), and 87.5% (7/8) of AFP cases identified in 2016, 2017, and 2018, respectively.

Viral isolation, molecular detection, and characterization of EVs

Virological investigations did not identify any PV in the AFP cases from 2016 to 2018. Following the WHO algorithm, samples from two cases (5.5%) showed CPE in RD cells but not in L20b cells, allowing us to exclude the presence of PV but confirming the presence of cultivable NPEVs that were characterized as E-25 (in 2016) and E-30 (in 2018).

NPEV RNA was detected in seven out of 36 AFP cases (19.4%), of which three were identified in 2016, two in 2017, and two in 2018. NPEV-positive AFP cases were detected mainly in July, irrespective of the year considered (Figure 1).

In 2016, the sequence analysis of three NPEV-positive samples revealed the presence of E-25 on fecal samples associated with a GBS case observed in June, one EV-D68 in respiratory samples associated with AFM in July (Pariani et al., 2017), and one EV-A71 on fecal and respiratory samples associated with brainstem encephalitis in December. The EV-D68 strain (accession number [AN]; MH118296) showed high similarity (nt. identity: 99.1-99.7%) with sequences of the B3 sub-clade identified in a 2016-AFM Dutch outbreak (Pariani et al., 2017). EV-A71 strain (AN; MH454234) shared high similarity (nt. identity: 98.4-98.7%) with sequences belonging to subgenogroup C1 also detected in an outbreak of brainstem encephalitis in 2016 in Spain (Casas-Alba et al., 2017).

Unfortunately, sequencing analysis of the two NPEV-positive samples identified in 2017 was abortive; both samples were

collected from two GBS cases identified in May and December, respectively.

In 2018, the molecular characterization of the NPEVs revealed the presence of EV-D68 and E-30, both associated with AFM identified in October and November, respectively. The EV-D68 strain (AN; MK790106) was similar (nt. identity: 97.8-98.2%) to sequences of the B2 sub-clade identified in the USA during an outbreak of AFM in 2016; while the E-30 strain (AN; MK815069) showed high similarity (nt. identity: 98.2-100.0%) to sequences belonging to group G, which have been circulating in Europe since 2016, dominating in 2018 (Broberg et al., 2018).

Discussion

Since the eradication of PV is still ongoing, reintroductions of this virus may pose a public health threat of major concern, especially for those countries within the current immigration routes, such as Italy. In this scenario, it is mandatory to strengthen AFPS at the regional and national level to rapidly detect any virus importation or emergence and enable a prompt public health response (Stefanelli et al., 2019). Although AFPS is the gold standard for PV control, Italy barely achieves the WHO target for AFPS performance indicators (WHO, 2016–2019). This could be related to the perception of AFPS as a low public health priority since Italy is a polio-free country and, consequently, PV became a neglected infection that is not considered a health hazard anymore (Stefanelli et al., 2019).

The Lombardy region, according to the WHO indicators for AFPS sensitivity, fully met the criteria of the non-polio AFP incidence ($\geq$1.00/100'000) in 2016 (1.1/100'000), while in 2017 (0.9/100'000) and 2018 (0.6/100'000), this indicator was not achieved. Despite not achieving the indicators' goals, overall, the AFPS results were adequate in Lombardy in terms of data quality and timeliness, completeness of case investigation ($\geq$80%), and completeness of clinical follow-ups. Also, performances in Lombardy showed an overall improvement of AFPS compared to the results obtained from 1997 to 2011 (Pellegrinelli et al., 2015) in the same surveyed area and those reached in other Italian regions (Fiore et al., 1999; Patti et al., 2000; Angelillo et al., 2001; D'Errico et al., 2008; Pellegrinelli et al., 2017a, 2017b; Palandri et al., 2020).

The AFP series presented in this study showed epidemiological characteristics similar to those reported in the current scientific literature, with the majority (60.5%) of patients being younger than five years. It is worthy of underlining that nearly 11% were hospitalized in intensive care units, and the remaining were reported by other wards (Patti et al., 2000; Angelillo et al., 2001; D'Errico et al., 2008; Pellegrinelli et al., 2017a, 2017b). The monthly distribution of AFP cases showed a peak in November and December when 44.4% of cases were notified, in line with what previously observed by others (Marx et al., 2000; Pellegrinelli et al., 2017a, 2017b), even if NPEV-positive AFP cases are detected mainly in July when NPEV are generally detected (Pellegrinelli et al., 2017a, 2017b; Broberg et al., 2018; Bubba et al., 2020). No wild PV was detected during this 3-year study, in line with what was observed at the national level, supporting the result that the routine PV immunization continued to achieve its coverage target every year.

After the interruption of PV transmission, GBS became the first cause of AFP (Angelillo et al., 2001; Barbadoro et al., 2020; Chen et al., 2020; D'Errico et al., 2008; Marx et al., 2000; Patti et al., 2000; Pellegrinelli et al., 2017a,b), along with other less frequently reported clinical manifestations, such as myopathy, unspecified causes of encephalitis, acute myelitis, and encephalomyelitis (Marx et al., 2000; Stefanelli et al., 2019). This was also observed
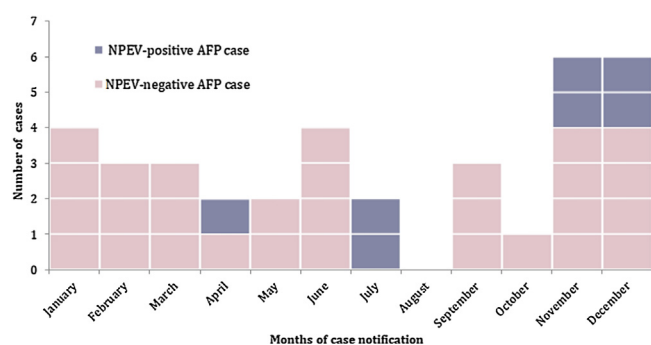

Figure 1. Temporal distribution of AFP cases and NPEVs detected in Lombardy (Northern Italy), from January 1, 2016, to December 31, 2018 in the framework of AFPS.

in our study, where GBS was the most common conclusive diagnosis, accounting for nearly 42% of AFP cases.

Unexpectedly, five children presented AFM, a newly defined, rare syndrome clinically distinct from AFP, that globally emerged in recent years, causing epidemics (Morens et al., 2019). In fact, since 2014 and in alternate years, an unexpected and unprecedented upsurge of severe respiratory infections and AFM outbreaks temporally associated with re-emergences of NPEV has raised concern about NPEV infections. In particular, EV-D68 recently emerged as a cause of AFM (Messacar et al., 2018), EV-A71 was implicated in outbreaks of brainstem encephalitis (Casas-Alba et al., 2017), and E-30 was associated with a wide meningitis-related outbreak with long-term sequelae in 2018 (Broberg et al., 2018).

Overall, 17.4% of AFP cases identified in this study resulted positive to NPEV, then molecularly characterized as E-25 (2016; n = 1), EV-D68 (2016; n = 1 – 2018; n = 1), EV-A71 (2016; n = 1), E-30 (2018; n = 1) and EV untypable (2017; n = 2). Interestingly, both molecular and epidemiological links observed in our AFP series overlapped what was observed in the same timeframe across Europe and the US. For instance, both EV-D68 and EV-A71 sequences detected in 2016 in our study showed high similarities with the B3-clade identified in a Dutch AFM outbreak in 2016 (Pariani et al., 2017) and to the subgenogroup C1, detected in a Spanish brainstem encephalitis epidemic in 2016 (Casas-Alba et al., 2017). This observation was also supported from the sequence analyses of NEPVs identified in 2018; in fact, EV-D68 identified in this study belonged to the B2 sub-clade, sharing high similarity with the US strains (Morens et al., 2019), and E-30 had a nucleotide identity of 98.2–100% with sequences belonging to group G1 that have been circulating in Europe since 2016 and dominating in 2018 (Broberg et al., 2018).

To date, no systematic NPEV hospital- and community-based surveillance systems are set up in Italy; thus, information on NPEV circulation is derived exclusively from observational studies, especially among children affected by severe conditions (Parisi et al., 2016; Piralla et al., 2014; Piralla et al., 2020). Nevertheless, with the emergence of several NPEVs - in particular EV-D68, EV-A71, and E-30 - involved in severe neurological manifestations (Bubba et al., 2020), AFPS has demonstrated renewed strength in continuing to provide epidemiological evidence to assist public health efforts (Kim et al., 2014; Suresh et al., 2018; Bubba et al., 2020; Roberts et al., 2020). Here, the unpredicted detection of emerging EV-D68, EV-A71, and E-30 strains sharing high nucleotide identity with viral strains involved in the European and US outbreaks identified in the same period, emphasizes the importance of monitoring NPEVs through AFPS to assess appropriate public health responses.

In conclusion, AFPS proved to be successful in PV surveillance and revealed wide NPEVs circulation linked with other concurrent European outbreaks. Continuing this surveillance activity is crucial to ensure that PV will be rapidly recognized if a new introduction would occur in polio-free countries; AFPS was demonstrated to be a helpful tool to monitor NPEVs circulation, and its enhancement should be encouraged as an early warning of future polio-like outbreaks.

Conflict of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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