Molecular Characterization of Coxsackievirus B5 Isolates from Sewage, Italy 2016–2017

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Abstract
Hereby, the partial Viral Protein 1 sequences of Coxsackievirus B5 (CV-B5) from sewage samples, collected in Italy from 2016 to 2017, were compared with those available in GenBank from clinical samples. Phylogenetic analysis highlighted: (I) the predominant circulation of CV-B5 genogroup B in Italy, and (II) the presence of two new sub-genogroups.

Keywords Coxsackievirus · CV-B5 · Sewage · Non-polio enteroviruses · Phylogenetic analysis · Polioviruses

Introduction
Environmental surveillance (ES) provides an early warning system for a possible introduction of poliovirus and, since 1996, is one of the activities of the Italian WHO Collaborative Reference and Research Center for Polio (2015). Meanwhile, ES examines the circulation and the spatio-temporal distribution of non-polio enteroviruses (NPEVs; Pons-Salort et al. 2018). In a recent study, our group analyzed more than 2800 sewage samples collected from 2009 to 2015. More than half of the samples were positive for NPEVs and Coxsackievirus B5 (CV-B5) being the most frequent serotype (Delogu et al. 2018).

Coxsackie B viruses are frequently associated with sporadic cases of neurological diseases, epidemics of meningitis, and chronic diseases such as cardiomyopathy and diabetes (Tracy and Gauntt 2008; Wikswo et al. 2009; Liu et al. 2014; Tao et al. 2014; Ma et al. 2013; Yao et al. 2017).

Henquell et al. (2013) described the genetic diversity of human CV-B5 clinical isolates with two main genogroups, A and B, detected worldwide. Genogroup A is characterized by sequential acquisition of nonsynonymous changes in residues exposed at the virus 5-fold axis; genogroup B is marked by the selection of three changes in the VP1 C-terminus from its first emergence.

The main aim of this study was to type the NPEVs identified from sewage samples collected from 2016 to 2017 in Italy and to compare the partial VP1 target gene of Italian CV-B5 strains in order to determine their sub-grouping.

Materials and Methods
Sewage samples were collected from 17 inlets of wastewater treatment plants (WWTPs) serving the urban areas of Naples, Bolzano, Parma, Sassari, Bari, Palermo, Catania,
**Results**

Overall, 423 sewage samples were collected, of which 244 were NPEV-positive by the cellular cytopathic effect on the RD cell line.

Half of the NPEV-positive samples (122/244) were selected for viral typing. In particular, for each Italian city participating in the surveillance we selected, in the period (2016–2017), half of their NPEV positive samples. The most frequent genotype was CV-B5 (26.2%, 32/122), followed by Echovirus (E)-6 (22.10%, 27/122), E-11 (12.30%; 15/122), and CV-B3 (11.5%, 14/122). The remaining 34 isolates belonged to 10 different genotypes: E-13 (7.38%), CVB-4 (5.74%), E-25 (4.92%), E-7 (4.10%), E-3 (1.64%), E-30 (0.82%), CVB-2 (0.82%), E-9 (0.82%), E-20 (0.82%), and E-19 (0.82%). One Sabin-like poliovirus type 3 strain was isolated from the WWTPs plant serving the urban area of Parma in October 2017.

Partial VP1 sequences (nt 2556 to 2874 of CV-B5 strain Faulkner complete genome) from 32 Italian CV-B5 strains, identified in sewage concentrates, were compared with 20 VP1 sequences representative of the 8 CV-B5 sub-genogroups described by Henquell et al. from clinical samples, available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), from 10 countries over a long time period (1977–2009, Table 1).

Figure 1 shows the genetic relationship among 52 VP1 sequences; moreover, the sequences of CV-B5 Faulkner and CV-B3 reference strains were also included.

Two VP1 Italian CV-B5 sequences, from sewage samples in the urban area of Naples, grouped with VP1 CV-B5 Faulkner reference strain within the genogroup A, being similar to the sub-genogroup A4 (Fig. 1). The remaining 30 Italian VP1 sequences, in the B branch together with VP1 sequences of genogroup B CV-B5 strains by Henquell et al., splitted into two novel sub-groups (B3 and B4). In fact, the genetic distance between the two newly described CV-B5 sub-groups (Italian samples) was estimated at 12.3%; while, B3 and B4 sub-groups differed from the sub-genogroups B described by Henquell et al. (sub-genogroups B0, B1 and B2) for 15.2 to 9.6 and for 15.5 to 9.3%, respectively. As a reference, the distance among sub-genogroups B described by Henquell et al. ranged from 6.9 to 13.1%. No
Table 1  Details of the CV-B5 Viral Protein 1 sequences used in the study

| ID       | Accession number | Genogroup/ sub-genogroup | Type of sample | Country of origin | City of isolation | Year of isolation | Month of isolation | Number of sampling per months |
|----------|------------------|---------------------------|----------------|-------------------|-------------------|-------------------|---------------------|-----------------------------|
| CF807S   | HF948028         | A0                        | Clinical       | FRA               | Not reported      | 1977              | Not reported        | Not applicable             |
| CF595    | HF948121         | A1                        | Clinical       | FRA               | Not reported      | 1999              | Not reported        | Not applicable             |
| 17036    | GU300063         | A1                        | Clinical       | NLD               | Not reported      | 1996              | Not reported        | Not applicable             |
| STU108   | HF948077         | A1                        | Clinical       | DEU               | Not reported      | 2004              | Not reported        | Not applicable             |
| P028     | GU300060         | A2                        | Clinical       | PAK               | Not reported      | 1990              | Not reported        | Not applicable             |
| CF19051  | HF948037         | A3                        | Clinical       | FRA               | Not reported      | 2006              | Not reported        | Not applicable             |
| LIM004   | HF948229         | A3                        | Clinical       | CYP               | Not reported      | 1996              | Not reported        | Not applicable             |
| GRE447   | HF948173         | A3                        | Clinical       | FRA               | Not reported      | 2003              | Not reported        | Not applicable             |
| CF186106 | HF948132         | A4                        | Clinical       | FRA               | Not reported      | 2005              | Not reported        | Not applicable             |
| COPT11098| HF948070         | A4                        | Clinical       | DNK               | Not reported      | 2008              | Not reported        | Not applicable             |
| ZY032    | GQ246515         | A4                        | Clinical       | CHN               | Not reported      | 2005              | Not reported        | Not applicable             |
| CF641    | HF948115         | B0                        | Clinical       | FRA               | Not reported      | 1979              | Not reported        | Not applicable             |
| 614      | GU300052         | B0                        | Clinical       | FIN               | Not reported      | 1984              | Not reported        | Not applicable             |
| 3939     | GU300050         | B0                        | Clinical       | USA               | Not reported      | 1982              | Not reported        | Not applicable             |
| BES1550  | HF948149         | B1                        | Clinical       | FRA               | Not reported      | 2000              | Not reported        | Not applicable             |
| 119229   | FJ868290         | B1                        | Clinical       | AUS               | Not reported      | 2004              | Not reported        | Not applicable             |
| COPT30075| HF948263         | B1                        | Clinical       | DNK               | Not reported      | 1993              | Not reported        | Not applicable             |
| BOL36    | HF948086         | B2                        | Clinical       | ITA               | Not reported      | 2008              | Not reported        | Not applicable             |
| NIC001   | HF948245         | B2                        | Clinical       | CYP               | Not reported      | 2009              | Not reported        | Not applicable             |
| STU6     | HF948275         | B2                        | Clinical       | DEU               | Not reported      | 2009              | Not reported        | Not applicable             |
| BZ-16-32 | MK517444         | B4                        | Environmental  | ITA               | Bolzano           | 2016              | September           | 2                           |
| BZ-16-36 | MK517473         | B4                        | Environmental  | ITA               | Bolzano           | 2016              | November            | 2                           |
| BZ-16-45 | MK517445         | B3                        | Environmental  | ITA               | Bolzano           | 2016              | December            | 2                           |
| BZ-17-02 | MK517446         | B3                        | Environmental  | ITA               | Bolzano           | 2017              | January             | 2                           |
| BZ-17-11 | MK517443         | B4                        | Environmental  | ITA               | Bolzano           | 2017              | March               | 2                           |
| BZ-17-23 | MK517447         | B4                        | Environmental  | ITA               | Bolzano           | 2017              | June                | 2                           |
| 1CAI-17-01| MK517470        | B3                        | Environmental  | ITA               | Catania           | 2017              | June                | 2                           |
| 1CAI-17-02| MK517448        | B3                        | Environmental  | ITA               | Catania           | 2017              | June                | 2                           |
| 1CAI-17-03| MK517449        | B3                        | Environmental  | ITA               | Catania           | 2017              | August              | 2                           |
| 1CAI-17-04| MK517450        | B3                        | Environmental  | ITA               | Catania           | 2017              | July                | 2                           |
| 1CAI-17-06| MK517451        | B3                        | Environmental  | ITA               | Catania           | 2017              | July                | 2                           |
| 2CAI-17-25| MK517452        | B3                        | Environmental  | ITA               | Catania           | 2017              | September           | 2                           |
| 2CAI-17-27| MK517453        | B4                        | Environmental  | ITA               | Catania           | 2017              | October             | 2                           |
| E276     | MK517454         | B4                        | Environmental  | ITA               | Parma             | 2017              | December            | 2                           |
| E277     | MK517455         | B4                        | Environmental  | ITA               | Parma             | 2017              | December            | 2                           |
| E278     | MK517457         | B4                        | Environmental  | ITA               | Parma             | 2017              | January             | 2                           |
| E279     | MK517458         | B3                        | Environmental  | ITA               | Parma             | 2017              | January             | 2                           |
| O277     | MK517456         | B4                        | Environmental  | ITA               | Parma             | 2017              | December            | 2                           |
| O278     | MK517464         | B4                        | Environmental  | ITA               | Parma             | 2017              | January             | 2                           |
| E281     | MK517459         | B3                        | Environmental  | ITA               | Parma             | 2017              | February            | 2                           |
| 1NA-16-18| MK517471         | B4                        | Environmental  | ITA               | Napoli            | 2016              | February            | 3                           |
| 2NA-16-21| MK517472         | A4                        | Environmental  | ITA               | Napoli            | 2016              | February            | 2                           |
| 1NA-16-23| MK517460         | B4                        | Environmental  | ITA               | Napoli            | 2016              | February            | 3                           |
| 2NA-16-28| MK517461         | A4                        | Environmental  | ITA               | Napoli            | 2016              | March               | 2                           |
| 1NA-16-29| MK517474         | B4                        | Environmental  | ITA               | Napoli            | 2016              | March               | 3                           |
| 1NA-17-50| MK517462         | B3                        | Environmental  | ITA               | Napoli            | 2017              | June                | 3                           |
| 1NA-17-58| MK517463         | B3                        | Environmental  | ITA               | Napoli            | 2017              | February            | 3                           |
| 2PA-16-79| MK517465         | B3                        | Environmental  | ITA               | Palermo           | 2016              | December            | 2                           |
Table 1 (continued)

| ID        | Accession number | Genogroup/sub-genogroup | Type of sample | Country of origin | City of isolation | Year of isolation | Month of isolation | Number of sampling per months |
|-----------|------------------|-------------------------|----------------|-------------------|-------------------|-------------------|----------------------|-------------------------------|
| 1PA-17-06 | MK517466         | B3                      | Environmental | ITA               | Palermo           | 2017              | January              | 2                             |
| 2PA-17-10 | MK517467         | B4                      | Environmental | ITA               | Palermo           | 2017              | February             | 2                             |
| 3PA-17-20 | MK517468         | B3                      | Environmental | ITA               | Palermo           | 2017              | March                | 1                             |
| SS-17-06  | MK517469         | B4                      | Environmental | ITA               | Sassari           | 2017              | March                | 2                             |

In italics the data published by Henquell et al. (2013)

Fig. 1 Phylogenetic tree based on the partial VP1 (nt 2556 to 2874 of CV-B5 strain Faulkner complete genome) nucleotide sequences. Trees were built using the maximum likelihood method (Kimura 2-parameter), and bootstrapped with 100 repetitions. Filled circles Italian sewages samples, open triangles genogroup B clinical samples described by Henquell et al. (2013), open squares genogroup A clinical samples described by Henquell et al. (2013)
relationships were found between the novel B sub-groups and geographic location of the sewage samples.

**Discussion**

ES, which is critical to support the global polio eradication endgame, permit to provide early detection of human enteric pathogens excreted with stools during an infection. Several studies reported a clear correlation between the isolation of enteroviruses in sewage, the isolation in humans, and clinical cases identified in the community (Nelson et al. 1967; Manor et al. 1999; Bisseux et al. 2018). All the NPEVs, here described, belonged to the species B, in agreement with what already found in sewage samples collected in Europe (Majumdar et al. 2018). Of note, it is the routine use of RD cell lines that follow the WHO protocol (2015), which favor for the isolation mainly of the EV species B (Majumdar et al. 2018).

The partial sequencing of VP1 was used to determine the serotype and to genetically analyze CV-B5 Italian strains detected in sewages versus CV-B5 strains from clinical samples (Henquell et al. 2013).

The phylogenetic analysis of a 319 nucleotides fragment of VP1 revealed a predominant circulation of genogroup B CV-B5 strains in Italy. This genogroup showed a low rate of evolution in the antigenic determinants over the last 50 years (Henquell et al. 2013).

However, phylogenetic analysis segregated the genogroup B Italian sequences into two relatively distant subgroups. The marked genetic divergence between the two Italian sequence-clusters and each of the three previously described sub-genogroups, suggests us to consider them as two novel CV-B5 sub-genogroups, namely B3 and B4. Due to the short sampling time period and high genetic conservation of VP1 region, the Italian CV-B5 sequences within sub-genogroups B3 and B4 resulted very similar with a low genetic distance (from 0.00 to 4.00%). In some cases (e.g., IDs E276, E277, E278) the VP1 sequences of the samples collected at the same site and at a short distance of time in the sampling were identical.

Hereby, the main findings are in agreement with what already described in Italy (Delogu et al.) in a more comprehensive sample size collected from 2009 to 2015. Moreover, the predominant circulation of CV-B5 of genogroup B was characterized by the presence of new subgroups evolving or being recently introduced in Italy.

As in many other European countries, also in Italy the real burden of EV disease can’t be affordably calculated due to many factors including viral diagnosis not always available for central nervous system diseases, pericarditis or cardiomyopathy, and for many other diseases like hand-foot-and-mouth disease or herpangina. Our results emphasize the need for improving national EV surveillance including genetic characterization of the virus isolated in Italy.

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**Compliance with Ethical Standards**

**Conflicts of interest** The authors declare that there are no conflicts of interest.

**Ethical Approval and Informed Consent** Not applicable.

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