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Research article

Molecular epidemiology of canine parvovirus type 2 in Sicily, southern Italy: A geographical island, an epidemiological continuum

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A B S T R A C T

Since it emerged as a major dog pathogen, canine parvovirus type 2 (CPV-2) has featured a remarkable genetic and phenotypic heterogeneity, whose biological, epidemiological, and clinical impact is still debated. The continuous monitoring of this pathogen is thus of pivotal importance. In the present study, the molecular epidemiology of CPV-2 in Sicily, southern Italy, has been updated by analysing 215 nearly complete sequences of the capsid protein VP2, obtained from rectal swabs/faeces or tissue samples collected between 2019 and 2022 from 346 dogs with suspected infectious gastrointestinal disease. The presence of the original CPV-2 type (4%) and CPV-2a (9%), CPV-2b (18%), or CPV-2c (69%) variants was documented. Over the years, we observed a decrease in the frequency of CPV-2a/-2b and a rapid increase of CPV-2c frequency, with a progressive replacement of the European lineage of CPV-2c by the Asian lineage.

The observed scenario, besides confirming epidemiological relevance of CPV-2, highlights the occurrence of antigenic variant shifts over time, with a trend toward the replacement of CPV-2a, CPV-2b, and the European lineage of CPV-2c by the emerging Asian CPV-2c lineage. The comparison with other Italian and international sequences suggests the occurrence of viral exchange with other Italian regions and different countries, although the directionality of such viral flows could not be often established with confidence. In several instances, potential CPV-2 introductions led to epidemiological dead ends. However, major, long-lasting clades were also identified, supporting successful infection establishment, local spreading, and evolution. These results, besides demonstrating the need for implementing more effective control measures to prevent viral introductions and minimize circulation, stress the relevance of routine monitoring activities as the only tool to effectively understand CPV-2 epidemiology and evolution, and develop adequate countermeasures.

1. Introduction

Canine parvovirus type 2 (CPV-2) is a non-enveloped single-stranded DNA virus, taxonomically included by the International Committee on Taxonomy of Viruses in the species *Protoparvovirus carnivoran1* (family *Parvoviridae*, subfamily *Parvovirinae*, genus *Protoparvovirus*) [1]. CPV-2 is the causative agent of an acute and often fatal disease of domestic dogs and wild carnivorans,

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characterized by severe gastroenteritis and lymphopenia. Nowadays, it is considered one of the main viral canine pathogens, threatening particularly young or non-immunised dogs [2].

The original type CPV-2, to date no longer circulating in the field and present only in some vaccines [3], was first identified as a canine pathogen in the late 1970s and has been soon replaced by three antigenic and genetic variants, namely CPV-2a, CPV-2b, and CPV-2c [4,5], currently distributed worldwide and circulating with different local frequencies. Indeed, CPV-2 has rapidly and progressively evolved, showing genomic substitution rates comparable to those of some RNA viruses [6], leading to the accumulation of point mutations and, during the years, the emergence of multiple lineages.

CPV-2 genome consists of a DNA molecule containing two gene cassettes including open reading frames (ORFs) encoding two non-structural proteins (NS1 and NS2) and two structural proteins (VP1 and VP2) [2]. VP2, the main constituent of the viral capsid, is the major determinant of host range and target of host immunity [7,8]. Many non-synonymous point mutations have occurred in the VP2 gene [9] and some protein residues are under strong diversifying selection pressure. One of them, residue VP2-426, is used to type CPV-2 variants (N in CPV-2a, D in CPV-2b, and E in CPV-2c). Other specific changes in the VP2 gene, detected in large different worldwide areas, allow us to track the evolution and spread of some lineages based on a combination of phenotypic features and spatial distribution [10,11].

In Italy, the evolution and spread of CPV-2 were evaluated several times [2,5,12,13], allowing to characterize the national CPV-2 distribution and, more recently, highlighting the detection of strains with new mutations [14,15]. Most of these studies are, however, based on convenience of sampling, and are characterized by limited sample size, origin, and type, with restricted information on CPV-2 circulation. Little data is also available for Sicily [13,16], a region in southern Italy and the largest and most populous island in the Mediterranean basin (25,711 km^2, 5 million inhabitants). The exact number of dogs living or moved to Sicily has not been determined, but a high number of free-roaming/stray dogs is present and animals are frequently imported from near or distant areas for adoption, creating favorable conditions for the introduction and diffusion of non-autochthonous viruses (https://www.salute.gov.it/portale/caniGatti/dettaglioContenutiCaniGatti.jsp?lingua=italiano&id=280&area=cani&menu=abbandono). For these reasons, there is a need to evaluate the impact of the spreading of CPV-2 in Sicily, assessing the genetic diversity of circulating viruses, and evaluating the viral epidemiology in the framework of the national, European, and global scenario. For this scope, a molecular epidemiological retrospective study was performed to analyse CPV-2 sequences obtained from samples collected from domestic dogs in Sicily between 2019 and 2022.

2. Material and methods

2.1. Study design

A retrospective study, based on CPV-2 sequences obtained from a collection of stored genomes, was performed. Samples were previously collected by private and public veterinary practitioners from 346 dogs showing clinical signs or gross lesions and with suspected infectious gastrointestinal disease and submitted to the Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" (Italy) for diagnostic purposes. All clinical samples, either obtained from live animals (rectal swabs or faeces, n = 127) or carcasses of dead dogs (n = 219), were collected in Sicily between 2019 and 2022 and, according to the laboratory routine, were screened for the presence of CPV-2 and other gastroenteric viruses, including canine adenovirus type 1 (CAdV-1) and type 2 (CAdV-2), canine distemper virus (CDV), canine coronavirus (CCoV), rotaviruses (RoVs), noroviruses (NoVs), by molecular assays.

2.2. Virus screening

DNA and RNA samples stored at −80 °C were previously extracted from supernatants obtained as described in Ref. [16], by using the DNeasy Blood & Tissue Kit (Qiagen S.p.A., Hilden, Germany) or the QIAmp Viral RNA Mini Kit (Qiagen S.p.A.), respectively, according to the manufacturer's instructions.

The presence of CPV-2 DNA was assessed by a diagnostic PCR assay, using a primer pair targeting the VP2 gene [17] and following a previously described protocol [18]. Extracted DNA and RNA were also tested by PCRs and RT-PCRs for CAdV-1/2, CDV, CCoV, and NoV, with primer pairs previously described [19–22]. A Real Time-PCR assay was performed to evaluate the presence of rotavirus RNA [23]. More details on gene targets, commercial kits, primers/probe, and expected amplicon sizes for the detected viruses are included in Supplementary Table S1.

Proportions of positive animals were compared using the Chi-square test and confidence on observed proportions is expressed as 95% normal intervals (confidence interval, CI). $P < 0.05$ was considered significant and Bonferroni correction was used for multiple proportion comparisons.

2.3. Sequence analysis

Sequencing of the near complete VP2 gene was carried out using the primer pairs developed by Battilani et al. [13], as previously described [24]. More details are included in Supplementary Table S1. Obtained amplicons were purified with Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK), and submitted to BMR Genomics srl (Padova, Italy) for direct Sanger sequencing with external and an additional internal primer [13]. Sequences were assembled and analysed using Geneious Prime 2022.0.2 (Biomatters, San Diego, CA, USA). Assembled nucleotide sequences were submitted to BLASTn [25] to search for the most related sequences in public databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on May

Table 1
Amino acid variations in VP2 gene sequences of analysed CPV-2 variants: divergent amino acids were bolded with a gray background.

						VP2 amino acid residues										
Viral strain	Acc.nr.	Variant[1]	N. Identical sequences	Sub-clade[2]	Year	5	13	139	183	267	324	370	371	418	426	440
2019PA10949	OR463522	CPV-2a	15	a	2019	A	P	V	M	F	**L**	Q	A	I	N	T
2019PA5124id436	OR463514	CPV-2a	3	a	2019	A	P	V	M	F	**L**	Q	A	I	N	**A**
2020CT1227	OR463518	CPV-2a	2	a	2019	A	P	**I**	M	F	**I**	Q	A	I	N	T
2019PA26796	OR463533	CPV-2b	34	d	2019	A	**S**	V	M	F	Y	Q	**G**	**T**	D	T
2019RG11304	OR463563	CPV-2b	2	d	2019	A	P	V	M	F	Y	Q	**G**	**T**	D	T
2022PA15678 idMeF	OR463607	CPV-2b (As)[3]	2	c	2022	**G**	P	V	M	**Y**	**I**	**R**	A	I	D	T
2019RG7696	OR463566	CPV-2c (Eu)	13	b	2019	A	P	V	M	F	Y	Q	A	I	E	T
2019PA30397	OR463579	CPV-2c (Eu)	7	b	2019	A	P	**I**	M	F	Y	Q	A	I	E	T
2019RG11305	OR463565	CPV-2c (Eu)	1	b	2019	A	**S**	V	M	F	Y	Q	A	I	E	T
2019PA28001	OR463616	CPV-2c (As)	114	NS	2019	A	P	V	M	**Y**	**I**	**R**	A	I	E	T
2020PA53415	OR463658	CPV-2c (As)	8	NS	2020	**G**	P	V	M	**Y**	**I**	**R**	A	I	E	T
2021PA43108idAki	OR463654	CPV-2c (As)	4	NS	2021	A	P	V	M	**Y**	**I**	**R**	A	I	E	**A**
2022PA19220idC1	OR463610	CPV-2c (As)	2	NS	2022	A	P	V	**I**	**Y**	**I**	**R**	A	I	E	T

[1] Eu: European CPV-2 lineage - As: Asian CPV-2 lineage.
[2] Referred to the sub-clade depicted in Fig. 2, NS: sub-clade not shown.
[3] Possible revertant of the Asian CPV-2 lineage.

04, 2023). Viral typing was based on the analysis of VP2 amino-acid (aa) residues discriminating the viral original type (CPV-2) and the CPV-2 variants [26]. CPV-2 sequences were clustered according to amino acid profiles and representative sequences included in Table 1. All sequences were submitted to the DDBJ/EMBL/GenBank databases (Supplementary Table S2).

2.4. Phylogenetic analysis

To evaluate the relationship with previously submitted international sequences, a complete dataset of VP2 was downloaded from GenBank, and the sequence name was annotated with collection host, county, and date when available. To reduce the computational burden and enhance graphical representation, a random subsample of 500 international sequences (dataset available upon reasonable request) was selected among unique ones. The Sicilian sequences obtained in the present study were aligned at the protein level to the reference ones using MUSCLE [27] implemented in TranslatorX [28] and then back-translated as nucleotides. The nearly full VP2 alignment was then subjected to Maximum Likelihood phylogenetic analysis using IQ-Tree [29] selecting the substitution model with the lowest Bayesian Information Criterion calculated using JmodelTest2 [30]. The reliability of inferred clades was assessed by performing 1000 bootstrap replicates. The obtained tree was edited and plotted using the *ggtree* library of R and related dependencies [31].

3. Results

3.1. Detection and typing of CPV-2

CPV-2 was detected in 230 out of 346 (66.5%, CI: 61.5–71.5%) dogs, from 114/127 (89.8%, CI: 84.5–95.1%) of the rectal swabs/faeces and 116/219 (53.0%, CI: 46.4–59.6%) of the tissue samples. CPV-2 single infections were observed in 190/230 dogs (82.6%, CI: 77.7–87.5%), whereas co-infections with other tested viruses were evident in 40/230 dogs (17.4%, CI: 12.5–22.3%) (Supplementary Table S3). Among identified co-infections, CCoV was the most frequently detected virus (n = 32 dogs), in mixed infection only with CPV-2 (28 dogs), with CPV-2 and NoV (3 dogs), or with CPV-2 and CAdV-1 (one dog). Two other viruses, NoV and CAdV-1, were co-detected with CPV-2 in 7 and 4 dogs, respectively.

All amplicons obtained from CPV-2 screening were successfully sequenced, but fourteen full VP2 sequences were excluded from subsequent analyses because of low quality (low amplification signals or poor-quality raw reads). A total of 215 sequences were thus successfully typed, revealing the presence of the original CPV-2 type (n = 8; 3.7%, CI: 1.2–6.2%), CPV-2a (n = 20; 9.3%, CI: 5.4–13.2%), CPV-2b (n = 38; 17.7%, CI: 12.6–22.8%), or CPV-2c (n = 149; 69.3%, CI: 63.1–75.5%). The rate of CPV-2-positive dogs in this study appeared constant during the considered timeframe, ranging between 61.6% and 75.8% (P = 0.3) (Fig. 1(a) and Supplementary Tables S3 and S4). Considering the three antigenic variants currently circulating, since 2019, the relative proportion of CPV-2a and CPV-2b variants decreased over time, while the frequency of detection of CPV-2c showed a proportional increase in the last two years, remaining the most detected variant during the whole studied period (Fig. 1(b) and Supplementary Table S4). Compared to 2019, the increase in relative frequency of CPV-2c was significant starting from 2021 ($P < 0.01$).

By discriminating the CPV-2c strains into European or Asian lineages (see below) and analysing their distribution during the years of detection, a rapid and statistically significant decrease in the relative frequency of the CPV-2c European lineage was noticed since 2020 ($P < 0.001$). At the same time, detections of the CPV-2c Asian lineage increased, completely replacing the European one in 2021 and 2022 (Fig. 1(b)–Supplementary Table S4).

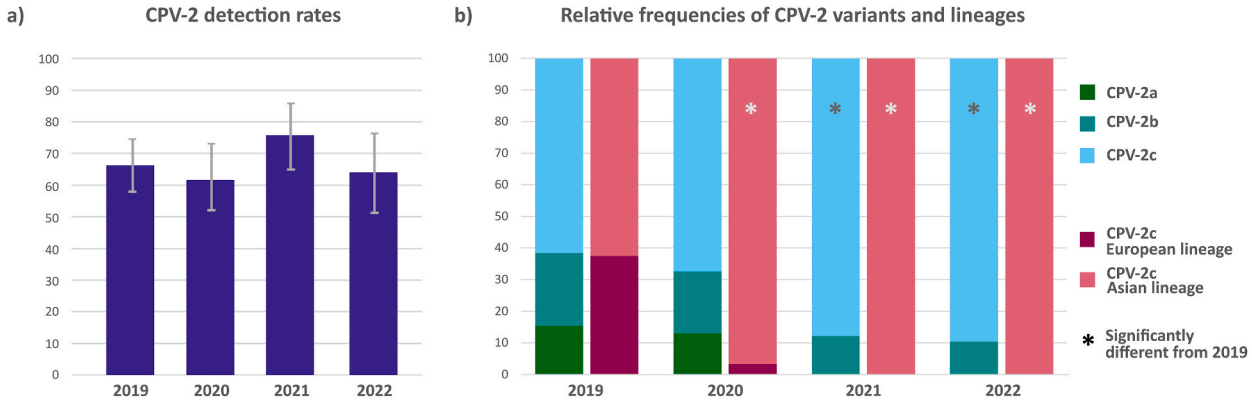

Fig. 1. Epidemiological trends observed in this study. (a) Proportions (%) of CPV-2 positive dogs during the years 2019–2022; vertical lines represent 95% IC. (b) Variations of the relative frequencies of the various CPV-2 variants and lineages over the years of analysis.

3.2. Phylogenetic analysis

In the phylogenetic tree (Fig. 2 and Supplementary Fig. S1), all CPV-2 sequences segregated mainly according to the geographical origin and the year of collection rather than to the antigenic variant. The phylogeny demonstrates that several Sicilian strains considered in the present study (reported in green), together with other Italian ones, were single lineages or part of small clades, suggestive of epidemiological dead ends. However, at least 4 major clusters, comprising a significant proportion of Sicilian strains, were also identified (Supplementary Figure S1 and Fig. 2). Among those, several CPV-2a strains clustered together with other closely related Italian CPV-2a sequences (Fig. 2(a)). However, foreign (of Asian origins) strains and a more divergent Italian group (including Sicilian strains 2020CT1227, accession number OR463516, and 2019PA6560, acc.nr. OR463517) were also included in this CPV-2a clade (Fig. 2(a)). CPV-2b strains herein reported were part of a monophyletic group together with other Italian strains only (Fig. 2(d)), similar to what occurred for several of the Sicilian strains classified in the European CPV-2c lineage (Fig. 2(b)). Sicilian Asian-like CPV-2c strains were highly related to each other and to other foreign strains previously reported, herein classified in the Asian CPV-2c lineage. Of note, two Sicilian CPV-2b strains (related to 2022PA15678idMeF strain, acc.nr. OR463607), fell within the CPV-2c Asian clade, together with a strain from Hungary (Fig. 2(c)). Altogether, these results evidenced the local/national co-circulation of several CPV-2 strains belonging to multiple lineages, although in the presence of several links with foreign countries.

3.3. Sequence analyses

According to sequence motifs identified in a previous study [32], all eight sequences classified as original CPV-2 type were considered as vaccinal strains. Sequences typed as CPV-2a, CPV-2b, or CPV-2c according to the amino acid residue discriminating the CPV-2 variants (VP2-426), were further characterized and grouped according to their amino acid sequences. In this way, 13 different unique sequences were identified, and a representative strain was chosen for each of them. The number of identical sequences for each representative is included in Table 1.

The sequences from this study showed a high pairwise nucleotide identity (99.8–98.6%). CPV-2a strains ids. 2019PA10949 and

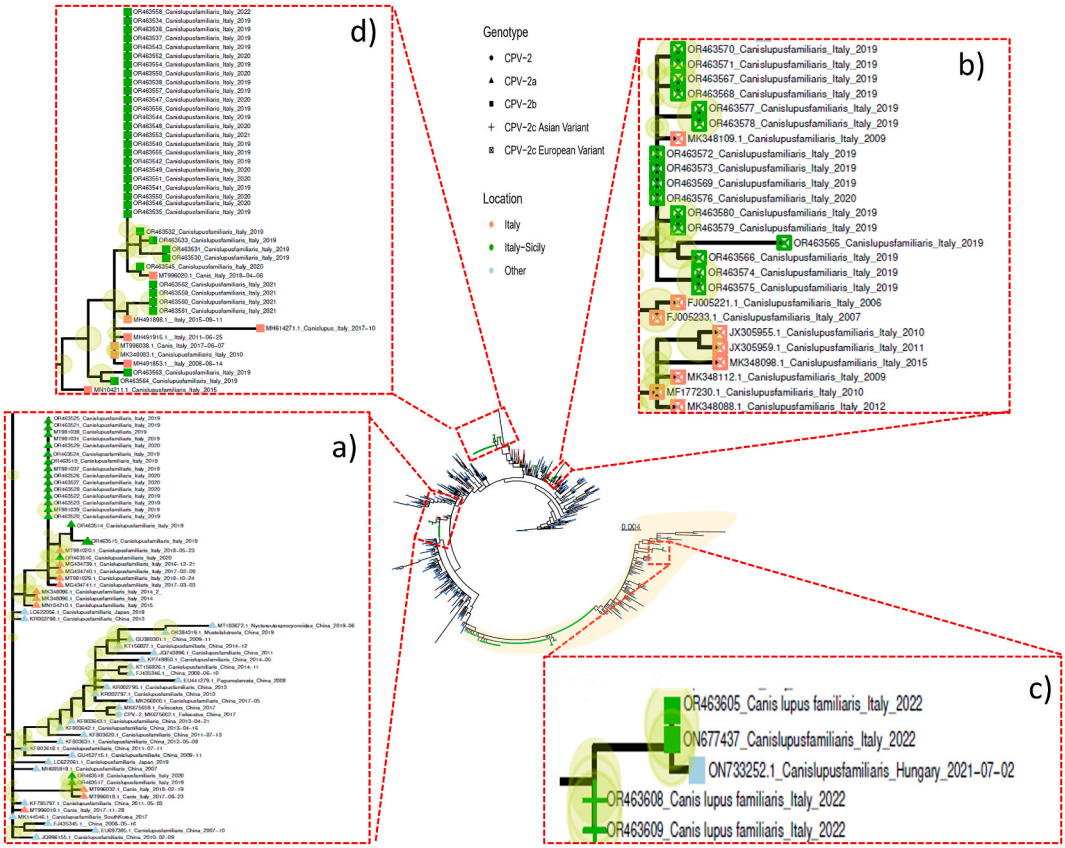

Fig. 2. Maximum likelihood phylogenetic tree based on the almost complete VP2 sequence of Sicilian strains plus a set of Italian and international representative CPV-2 strains. Antigenic variants, defined based on marker amino acids, have been coded with different tip shapes while the location where samples were collected has been color-coded. The bootstrap support values have been depicted through shaded circles overlapping the corresponding node and whose size is proportional to the estimated value. Strains classified as Asian CPV-2c lineage have been emphasized/highlighted with a yellow background. Further details are reported in Supplementary Fig. S1.

2019PA5124id436 showed the highest nucleotide identity (>99.94%) with CPV-2a strains previously detected in Sicily since 2016 [18,33], while strain 2020 CT 1227 was identical to a strain collected in 2018 from a cat in Veneto (a region in northern Italy) [34], suggesting an occasional and incursive introduction.

Within the CPV-2b variant, strain 2019PA26796 showed the highest nucleotide identity (99.89%) with CPV-2b strains previously detected in Sicily in 2018 (accession number MT981027 [33]; and in 2020 (ON479068 [32]; strain 2019RG11304 with CPV-2b strains (99.94%) detected in Apulia (another region in southern Italy) in 2013 (MH491875; [12]) and in Emilia-Romagna (north-eastern Italy) in 2016 (MK348102; [13]); strain 2022PA15678idMeF showed the highest identities with CPV-2b strains detected on January 2022 in Sicily (100%; ON677437; [15]) and in July 2021 in Hungary (99.94%; ON733252; [35]). Although these latter strains, based on the VP2-426 residue, have been typed as CPV-2b, according to the phylogeny and the analysis of other aa residues, they could be considered as Asian CPV-2c likely revertant strains, showing how genotyping based exclusively on residue 426 is reductive. These results evidenced the spread of closely related but distinct CPV-2b strains within the same territory, with the sudden and sporadic recent detection of strains related to east-European and Asian strains.

Sequences of CPV-2c strains showed relatedness with the CPV-2c strains circulating in Europe (herein defined as CPV-2c "European" lineage) identified for the first time in 2000 [5] or with a CPV-2c originated in Asia (defined as CPV-2c "Asian" lineage), first evidenced in Europe only in 2017 [14]. Strains ids. 2019RG11305, 2019RG7696, and 2019PA30397 (representative of 21/149 CPV-2c strains) showed the highest nucleotide identities with CPV-2c "European" strains detected in Sicily in 2000 (99.66%; FJ222821; [36]) and in 2009 (99.94–99.89%; KU508407), and in the U.S.A. in 2010 from a *Puma concolor* (99.89%; JX475260; [37]. In contrast, strains 2019PA28001, 2021PA43108 idAki, and 2022PA19220idC1 (representative of 120/149 CPV-2c strains) showed 99.83–100% nucleotide identities with a CPV-2c "Asian" strain detected in Sicily in 2018 (MK806280 [38]; and, in the same period, in Asian countries (China, in 2016–2019; South Korea, in 2019) and in Nigeria, in 2018; strain id. 2020PA53415 showed 100% nucleotide identity with a CPV-2c "Asian-like" strain detected in Sicily in 2017 (MF510157; [14]) and other 131 sequences of CPV-2c strains collected from dogs in Asian countries (China, 2017–2021; Thailand, 2016 and 2020; Indonesia, 2013; Vietnam, 2017–2020; South Korea, 2017) or other continents (Nigeria, 2018; Canada, 2018; Romania, 2019; Ethiopia, 2021; India, 2022), from two dogs transferred from Sicily to Emilia Romagna region in 2019 (OM892843-4; [39]), from a cat in Thailand in 2016 (MH711902) and in China in 2019 (MT270587), and from a pangolin (*Manis pentadactyla*) in China in 2020 (OP208805).

Sequence analysis revealed amino acid changes in all CPV-2 variants (summarized in Table 1), some of which were convergent among different variants (CPV-2b/CPV-2c: P13S; CPV-2a/CPV-2c: V139I; CPV-2a/CPV-2c: Y324I; CPV-2a/CPV-2c: T440A), while others were unique (CPV-2c: M183I; CPV-2b: A371G, I418T). Among these, M183I was common only to a CPV-2c strain detected in Vietnam in 2017 (MK357734), while the other two changes (A371G, I418T) were common to CPV-2b strains collected in other Italian regions since 2008 [12,13]. Within the CPV-2b variant, a divergence was observed at the VP2-13 aa residue, with 89% of analysed CPV-2b strains showing S at this residue. Some amino acid changes discriminated between CPV-2c lineages of "European" or "Asian" origins (VP2-5 A/G, −267Y, −324I, −370R).

4. Discussion and conclusions

Soon after its first evidence in the late 1970s [40], CPV-2 rapidly spread worldwide and is still considered the main viral cause of gastroenteric infection in canine species, particularly in younger dogs, although the wide use of vaccines has substantially contributed to the control of this infection [2]. During the last four decades, an increasing number of studies investigated its spread, epidemiology, and evolution, leading to a more in-depth characterization of CPV-2 genomic heterogenicity.

In Italy, somewhat differently to most other European countries, a considerable amount of literature has contributed during the years to outline the evolution and spread of CPV-2 in domestic dogs [2,5,12,13,26,41–43]. Overall, several pieces of evidence suggested a continuous change in the occurrence and distribution of classical and new CPV-2 lineages, that need to be constantly monitored. Under this light, the present study provides a rational analysis of the distribution of CPV-2 genetic lineages and variants in Sicily, in southern Italy, during the last four years, to track the spreading dynamics of CPV-2 strains and analyse their epidemiological patterns. Indeed, the continuous molecular monitoring in this region already allowed to detect, for the first time, novel CPV-2 strains in Italy or Europe [14,15,18]. Nonetheless, the relevance of all detected CPV-2 lineages has not yet been evaluated in detail.

Most of the current studies on CPV-2 showed a consensus on naming the CPV-2 circulating variants as CPV-2a, CPV-2b, and CPV-2c, even though they do not correspond to phylogenetically defined clades. Nevertheless, during the course of the years, molecular typing based on VP2 sequences allowed a rapid and more informative characterization, granting a high-resolution comparison of CPV-2 strains, and improving the spatial tracing and evolutionary studies. For these reasons, the commonly accepted definition for the CPV-2 variants was used for this study, in addition to the use of phylogenetic analysis and the definition of other relevant VP2 residues that allowed a more in-depth description of locally circulating lineages.

CPV-2 variants, excluding vaccinal strains, were observed as a leading cause of viral gastroenteric infection of dogs in Sicily, infecting 66.4% of tested dogs, more often as a single infecting viral agent (79.1% of CPV-2 positive dogs). In mixed infections, CCoV was the most frequently detected virus (80% of dogs with mixed infection). Previous studies [44–46], conducted in southern and insular Italy, agree on the great importance of CPV-2 as an agent of viral gastroenteritis in dogs, considering that it has been found with a prevalence of 58–87%, followed by the CCoV, which was detected with sensibly lower rates (18–31%). Unfortunately, the lack of other more recent studies on CPV-2 and other gastroenteric viral pathogens prevented further comparison on the epidemiology of CPV-2 in other Italian regions. The high prevalence of CPV-2 infection highlighted in this study suggests the relevance of CPV-2 as the cause of enteric disease, with a limited or sometimes negligible role of other tested pathogens. A similar observation on the greater pathogenic role of CPV-2, compared to CCoV, was already suggested by Alves et al. [47]. These results underline the need of improving

further regional efforts to control this infection through vaccination [48,49]. Notably, vaccine-derived CPV-2 strains were detected in 1–3 samples per year, ranging between 1 and 9% of the positive samples. The occasional occurrence of vaccinal CPV-2 strains, as already observed [32], underlines the need to molecularly characterize the identified viruses, in order to discriminate between field and vaccinal strains since PCR-positive results are not necessarily indicative of an active infection by a field strain.

The characterization of the obtained Sicilian strains and their comparison with the previously obtained sequences revealed an overall complex connection network involving both other Italian regions and foreign countries. The present study revealed that the Italian strains were interspersed in the phylogenetic tree, suggesting the occurrence of multiple introductions from different national or international areas, and other European countries and Asia were the most common sources. In several instances, Italian and Sicilian strains were single lineages or part of small clades, suggestive of epidemiological dead ends. However, at least 4 main clades, classified in the so-called antigenic variants CVP-2a, CPV-2b, European CPV-2c, and Asian CPV-2c were identified, including, with minor exceptions, only Sicilian and Italian sequences, with an overall tendency of Sicilian strains to cluster together within broader Italian clades. The frequent localization of Sicilian strains on terminal branches might suggest CPV-2 introduction from other Italian regions. However, several exceptions were also present, and the lack of a systematic sampling prevents definitive conclusions. The reported pattern suggests that after its introduction in Italy, the virus was in some instances able to persist and spread within country borders, progressively evolving. Further studies would be useful to investigate the reason behind the differential success and fitness of individual CPV-2 strains and lineages, as well as the specific introduction and spreading patterns within Italy, with a particular focus on the directionality of viral flux from and/or to Sicily.

Considering the national scenario, data referred to the last decade showed a relatively higher prevalence of CPV-2a in continental Italy, followed by CPV-2b and CPV-2c [12,13,44,50], with some differences on regional bases. An exception is Sardinia, where a higher prevalence of CPV-2b, followed by CPV-2a and CPV-2c, was observed [43,46]. Early typing of circulating CPV-2 strains in Sicily [16], performed on samples collected in 2009–2015, revealed a high CPV-2c prevalence (79.56%), although other variants were not analysed in detail. Specific variants have been detected in Sicily in 2017–2018 [14,18,33], with some apparent spreading within the regional territory [38]. These studies were based on a limited number of CPV-2 sequences, without investigating the full spectrum of circulating strains. The occurrence of previously unreported CPV-2 strains and lineages suggested the need for a systematic analysis of all CPV-2-positive dogs, to evaluate their impact and frequency over time and, above all, to assess the genetic relationships with currently known national or international viruses.

Already in 2019, CPV-2c relative frequency was higher (62%) than CPV-2b (23%) and CPV-2a (15%): CPV-2a presence dropped in 2020 and was no longer detected in 2021–2022; similarly, CPV-2b frequency (initially 23%) halved in the last two years. Conversely, CPV-2c relative frequency progressively increased, reaching 90% in 2022. Another striking finding was the rapid and, since 2021, total replacement of the European CPV-2c lineage by the Asian one. The European CPV-2c lineage was detected for the first time in Sicily in 2000 [5] and rapidly spread throughout Europe and other continents, particularly in South America [10]. With the only exception for three CPV-2c strains, genetically related to CPV-2c European strains, from samples collected in Vietnam in 2002 (strain HNI-4-1, acc. nr. AB120727; [51]) and in China in 2009 (isolates 06/09, GU380305, and 08/09, GU380305; [52]), CPV-2c has not been detected in Asia until 2013. Simultaneously, a new CPV-2c variant, also defined as “Asian” or “of Asian origin” started to rapidly spread within Asian countries [53]. In subsequent years, this Asian lineage has been detected also in non-Asian countries, such as Italy [14] and other European countries [50,54], Africa [24,55–57], and North America [58]. As observed in this study, since 2020, a rapid trend of transition from CPV-2a/CPV-2b to CPV-2c predominance was documented also in different Asian countries [59–63].

Molecular markers of CPV-2 variants allowed us to observe the absence of circulation of CPV-2a with the VP2-324L signature, first detected in 2018 in the same region [18], and the occasional detection of single specific CPV-2a strains (with VP2–139I and −324I) related to viruses collected in north-eastern Italy [12,34,50] and Hungary [64]. While a low circulation of CPV-2a lineages is reported in Sicily, this is different from what was reported for continental Italy. Differently, two CPV-2b lineages (VP2–13 P/S) continue to co-exist in Sicily, with different relative frequencies (6% *versus* 94%). Similar to a previous study [13], the prevalent CPV-2b lineage in Italy shows VP2–13S, with limited analogous sequences with this mutation collected from domestic dogs in Apulia (MH491875), Emilia-Romagna (MK348102), and Veneto (MN104197) or from a beech marten (*Martes foina*) (MT353763), a European badger (*Meles Meles*) (MT353762), and a wolf (*Canis lupus italicus*) (MT353761) in Abruzzi region. Interestingly, the residue VP2–13P was observed also in older CPV-2b strains collected in the 1990s or 1980s mainly from wild animals [6,65], while in the present study, it was evidenced in only one animal. Similarities shared by these CPV-2b strains found in domestic animals and wildlife, spanning a wide area from southern (Apulia), and central (Abruzzi), to northern (Emilia-Romagna, Veneto) Italian regions, suggest the need for further epidemiological evaluations.

Strains related to id. 2022 PA 15678 idMf showed high identity and the same aa pattern with a CPV-2b strain collected in Hungary in July 2021 (ON733252; [35]), a few months before their first evidence in Italy [15]. However, according to the other amino acid pattern and phylogenetic analysis, these CPV-2b strains are part of the CPV-2c Asian lineage and are likely phenotypically revertant strains [53]. Nonetheless, their spatial-temporal origin is still unclear, although the presence in Hungary in the previous months could suggest their potential spread among different European countries.

The circulation of different but closely related Asian CPV-2c strains in Sicily was also proven. Interestingly, this CPV-2 mutant has been detected only recently in samples of dogs from north-eastern Italy (Friuli Venezia Giulia), in 2013 and 2015, and from Hungary in 2014 [50]. This geographical pattern might suggest a route of incursive introduction between eastern Europe and north-eastern Italy, presumably related to dog transport. Similarly, the same CPV-2c variant has been observed in dogs transported from Sicily to northern Italy for adoption [39]. All these occurrences, along with the first description of the Asian CPV-2c strain in Europe [14], underline how long-distance transport of dogs could be a major driver for the emergence and spread of different CPV-2 variants to new areas.

A debate on the effectiveness of vaccines currently in use still remains open and stimulates further studies based on specific

laboratory tests. Indeed, the ability of classical or recombinant available vaccines to protect towards the classical CPV-2c variant has been already proven [66–69]. However, these studies are based on the "older" variants and, as of today, a specific study investigating the efficacy of vaccines against CPV-2c Asian lineage strains has not yet been performed. Indeed, the recent and rapidly progressive widespread of the CPV-2c Asian lineage among the Asian canine population raised concerns and questions on the effectiveness of vaccines towards these novel strains, since specific amino acid changes have been hypothesized as potentially responsible for vaccine failure [70]. The current spread of this CPV-2c Asian lineage in other continents [53] or the rapid changes of circulating CPV-2 variants in specific geographic areas, as also observed in this study, further stress the need to provide an answer to these questions. In order to highlight other potential factors for vaccine failure towards CPV-2 [71,72], viral strains with specific amino acid patterns can be then suggested as next potential targets for further studies with specific and more conclusive serological tests.

In conclusion, this study analysed the viral distribution of CPV-2 in an insular Mediterranean area of southern Italy, underlining how plastic and evolving the current epidemiological scenario is and outlining the shifts in the frequency of the different variants in the same geographical environment over a few years. This epidemiological scenario could mirror a wider, national and/or international, spatial occurrence.

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Ethics statement

Review and/or approval by an ethics committee was not needed for this study since no experiments on live animals were performed; the study was carried out using remaining material obtained during routine clinical diagnostic activity.

Data availability statement

All sequences are included in the DDBJ/EMBL/GenBank databases under accession numbers OR463514 - OR463704, MT981031 - MT981039, MK806282 - MK806285, ON677437. The dataset for the phylogenetic tree presented in this study can be obtained from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Francesco Mira: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giorgia Schirò:** Writing – original draft, Investigation, Formal analysis, Data curation. **Giovanni Franzo:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Marta Canuti:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Giuseppa Purpari:** Supervision, Project administration, Methodology, Investigation, Formal analysis. **Elisabetta Giudice:** Supervision, Methodology, Investigation, Data curation. **Nicola Decaro:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **Domenico Vicari:** Supervision, Resources, Investigation, Formal analysis. **Francesco Antoci:** Supervision, Resources, Investigation, Formal analysis. **Calogero Castronovo:** Investigation, Formal analysis. **Annalisa Guercio:** Writing – review & editing, Validation, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nicola Decaro reports financial support was provided by European Union.

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Appendix A. Supplementary data

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