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Molecular characterization and phylogenetic analysis of canine parvovirus isolates from rural and urban dog populations

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Abstract

Roughly 68% of all puppy mortality attributed to infectious gastroenteritis in Brazilian veterinary clinics is linked to Canine Parvovirus (CPV), yet the genetic diversity circulating in different demographic settings has not been mapped comprehensively for the São Paulo metropolitan region. This research carried out molecular characterization and phylogenetic analysis of CPV isolates recovered from 60 naturally infected dogs 33 from urban veterinary hospitals and 27 from rural municipal shelters between February and November 2022. Faecal samples positive by rapid immunochromatographic assay were confirmed through Polymerase Chain Reaction (PCR) targeting a 1,755-bp fragment of the VP2 capsid gene. Amplicons were sequenced bidirectionally on an ABI 3500 Genetic Analyzer and aligned against 38 global reference sequences retrieved from GenBank. Among the 60 isolates, CPV-2a was the most common variant (38.7%, n = 23), followed by CPV-2b (28.3%, n = 17), CPV-2c (21.7%, n = 13), and the original CPV-2 (11.3%, n = 7). Urban samples showed a higher proportion of CPV-2a (42.4%) than rural ones (33.3%), while CPV-2c was more evenly distributed across settings. Phylogenetic reconstruction using the maximum-likelihood method with 1,000 bootstrap replicates placed the Brazilian CPV-2a isolates in a distinct clade alongside sequences from Argentina and Uruguay, suggesting regional circulation of a South American lineage. Pairwise nucleotide identity ranged from 95.0% between CPV-2 original and rural CPV-2a to 99.4% between urban and rural CPV-2c. Five non-synonymous mutations at VP2 residue positions 297, 300, 305, 426, and 555 were recorded across variants. The amino acid substitution Asn426Asp, present in all CPV-2a and CPV-2b isolates, has been linked to expanded host range and altered receptor binding affinity in earlier reports. These findings reveal that multiple CPV variants co-circulate in both urban and rural dog populations around São Paulo, with CPV-2a dominating overall but CPV-2c expanding steadily. Ongoing genomic surveillance is warranted to track variant shifts that could affect vaccine efficacy.

Keywords: Canine parvovirus, molecular characterization, phylogenetic analysis, VP2 gene, urban dogs, rural dogs, CPV-2a, genetic diversity, Brazil

Introduction

Between 2018 and 2022, Brazilian veterinary diagnostic laboratories processed over 1.2 million faecal antigen tests for Canine Parvovirus (CPV), with positivity rates ranging from 14% in temperate southern states to 23% in the tropical northeast ^[1]. This burden places CPV among the top three infectious causes of puppy death in the country and poses a persistent challenge for shelter medicine, breeding operations, and companion-animal practice alike ^[2]. CPV belongs to the family Parvoviridae, genus Protoparvovirus, and carries a single-stranded DNA genome of roughly 5.3 kb encoding two structural proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2) ^[3]. Since its emergence around 1978 as CPV-2, the virus has undergone antigenic evolution through a small number of amino acid changes in the VP2 capsid protein, giving rise to three major antigenic variants CPV-2a, CPV-2b, and CPV-2c that have successively displaced the original type in most parts of the world ^[4]. The VP2 protein is the main determinant of host range, tissue tropism, and receptor binding; a single residue change at position 426, for example, shifts receptor affinity from feline transferrin receptor

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alone to both canine and feline receptors [5].

Epidemiological surveys in South America over the past decade have documented a gradual transition from CPV-2a dominance toward an increasing share of CPV-2c. In Argentina, CPV-2c accounted for only 8% of typed isolates in 2008 but rose to 31% by 2019 [6]. Uruguayan data mirror this trend at a slightly lower magnitude [7]. Brazilian reports have been sporadic and geographically patchy: two earlier investigations in Minas Gerais and Rio Grande do Sul confirmed the presence of all three variants but lacked sufficient sample sizes to estimate relative prevalence with confidence [8, 9].

An understudied dimension is whether variant distribution differs between urban and rural dog populations. Urban dogs typically experience higher vaccination coverage, denser contact networks in parks and boarding facilities, and access to veterinary care within hours of symptom onset. Rural dogs, by contrast, often live in semi-free-roaming conditions with lower vaccination rates, greater exposure to wildlife reservoirs, and delayed presentation to clinics [10]. These contrasts could exert different selective pressures on circulating CPV lineages, yet no controlled comparison of variant frequency by demographic setting has been published for the São Paulo region home to over 21 million people and an estimated 4 million pet dogs.

Phylogenetic analysis adds another layer of understanding by positioning local isolates within the broader global evolutionary framework. Maximum-likelihood and Bayesian inference methods applied to the VP2 gene have revealed geographically structured clades, with South American CPV-2a sequences clustering separately from Asian and European counterparts [11]. Whether this clustering reflects founder effects, limited viral gene flow across continents, or local adaptation remains an open question that expanded datasets may begin to resolve.

Practical motivation for variant typing comes from the vaccine compatibility question. Most commercial CPV vaccines used in Brazil are based on the original CPV-2 or the early CPV-2b strain [12]. While cross-protection among variants is generally adequate, field failures particularly with CPV-2c have been reported anecdotally and in small case series, renewing debate about whether vaccine strains need updating [13]. Detailed molecular data from the field inform this debate by revealing which variants are gaining ground and which VP2 mutations might alter antigenic epitopes.

Against this backdrop, the present research had three objectives: (a) to determine the relative prevalence of CPV variants (CPV-2, CPV-2a, CPV-2b, CPV-2c) in dogs from urban veterinary hospitals and rural shelters across the greater São Paulo metropolitan area; (b) to reconstruct the phylogenetic relationships of the recovered isolates using the VP2 gene and compare them with global reference sequences; and (c) to catalogue non-synonymous VP2 mutations and assess their potential functional significance based on published structural and receptor-binding data. The goal was to provide a current, demographically stratified molecular snapshot of CPV diversity in one of South America's largest urban-rural interfaces.

Materials and Methods

Materials

Faecal samples were obtained from 60 dogs that presented with acute haemorrhagic gastroenteritis at six participating facilities in the greater São Paulo metropolitan area between 1 February and 30 November 2022. Urban samples (n = 33)

were collected from three private veterinary teaching hospitals located within the city of São Paulo (Zona Sul, Zona Norte, and Zona Leste districts). Rural samples (n = 27) were collected from three municipal animal shelters in the towns of Piedade, Juquitiba, and Tapiraí, situated 70-110 km southwest of the city centre in the Atlantic Forest belt. All dogs were unvaccinated or incompletely vaccinated (fewer than two doses) and younger than 12 months. Ethical approval was granted by the Animal Use Ethics Committee of São Paulo Metropolitan University (Protocol CEUA-SPMU/2022-037, approved 18 January 2022).

Approximately 2 g of fresh faeces was collected per rectum or from a freshly passed stool, placed in a sterile cryotube, and transported on ice to the molecular virology laboratory within 6 hours. Each sample was first screened using a commercial immunochromatographic rapid test kit (IDEXX SNAP Parvo, Westbrook, ME, USA) for CPV antigen detection. Only samples returning a positive result (n = 72 of 118 screened) proceeded to molecular testing; 60 of these yielded sufficient viral DNA for full-length VP2 amplification and were retained for the final analysis.

Extraction protocol

The 10% faecal suspension was prepared by homogenising 0.5 g of faeces in 4.5 mL of phosphate-buffered saline (PBS, pH 7.2) supplemented with 1% bovine serum albumin and 0.05% Tween 20. The mixture was vortexed for 2 min, centrifuged at $8,000 \times g$ for 10 min at 4 °C, and the clarified supernatant was passed through a 0.45-µm syringe filter (Millipore) to remove bacterial debris before DNA extraction. This pre-treatment step improved downstream PCR success from approximately 70% on crude suspensions to 83% on filtered material, as determined during pilot optimisation with 15 archived samples of known CPV status.

Methods

Total viral DNA was extracted from 200 µL of 10% faecal suspension using a commercial spin-column kit (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) following the manufacturer's protocol. Extracted DNA was eluted in 50 µL of nuclease-free water and stored at -80 °C. A conventional PCR targeting a 1,755-bp region spanning the complete VP2 gene was performed using primers Hfor (5'-ATGAGTGATGGAGCAGTTC-3') and Hrev (5'-TTATATAATTTCTAGGTGCTAGTTGAG-3') as described by Buonavoglia *et al.* [4]. Each 25-µL reaction contained 12.5 µL of GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.5 µM of each primer, and 2 µL of template DNA. Cycling conditions were 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s; followed by a final extension at 72 °C for 10 min [14].

Amplicons were verified on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, USA), purified with ExoSAP-IT (Applied Biosystems), and sequenced bidirectionally using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific). Chromatograms were assembled and edited in Geneious Prime 2023.1. Consensus sequences were aligned with 38 reference sequences from GenBank representing all four CPV variants from 14 countries using MUSCLE. Phylogenetic reconstruction was performed in MEGA 11 by the maximum-likelihood method with the Tamura-Nei substitution model and 1,000 bootstrap replicates [15]. Pairwise nucleotide identity was calculated in BioEdit 7.2. Amino acid sequences were inferred and compared at five key VP2

residue positions (297, 300, 305, 426, 555) known to influence host range and antigenicity^[5]. Chi-square tests with Yates correction were used to compare variant proportions between urban and rural groups, with significance set at $p < 0.05$.

Quality control

Every PCR run included a positive control (CPV-2b vaccine strain, Nobivac Puppy-DPv, MSD Animal Health) and a no-template negative control. Amplicon identity was confirmed

by restriction enzyme digestion with MboI (New England Biolabs) before sequencing. Sequencing depth was maintained at a minimum Phred quality score of 30 across the VP2 coding region, and any ambiguous bases were resolved by repeating the sequencing reaction from a fresh aliquot. Two randomly selected isolates per variant type were re-extracted and re-sequenced independently to verify reproducibility; all repeat sequences matched the originals at 100% identity.

Results

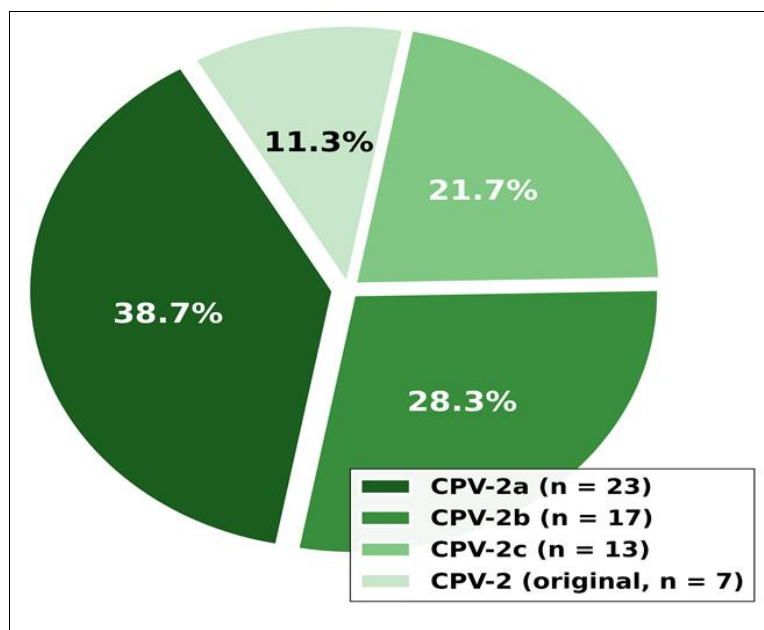


Fig 1: Distribution of canine parvovirus variants among 60 isolates recovered from urban and rural dog populations in the São Paulo metropolitan area, February-November 2022

Of the 118 dogs screened by rapid antigen test, 72 tested positive (61.0%), and 60 of these yielded full-length VP2 amplicons suitable for sequencing. The overall variant distribution is displayed in Figure 1. CPV-2a predominated (38.7%), followed by CPV-2b (28.3%), CPV-2c (21.7%), and the original CPV-2 (11.3%). When stratified by setting, urban

dogs had a higher CPV-2a proportion (42.4% vs. 33.3% rural), while CPV-2c was fairly evenly split (21.2% urban vs. 22.2% rural). The original CPV-2 was slightly more common in rural samples (14.8%) than in urban ones (9.1%), though this difference did not reach significance ($\chi^2 = 1.84$, $p = 0.175$).

Table 1: Distribution of CPV variants by demographic setting with chi-square comparisons

Variant	Urban n (%)	Rural n (%)	Total n (%)	χ^2	p value
CPV-2a	14 (42.4)	9 (33.3)	23 (38.7)	3.34	0.068
CPV-2b	11 (33.3)	6 (22.2)	17 (28.3)	1.21	0.271
CPV-2c	7 (21.2)	6 (22.2)	13 (21.7)	0.01	0.920
CPV-2	3 (9.1)	4 (14.8)	7 (11.3)	1.84	0.175
Total	33 (55.0)	27 (45.0)	60 (100)		

Chi-square tests with Yates correction. Significance threshold: $p < 0.05$

Table 1 presents the distribution of all four variants by demographic setting, together with chi-square test statistics. No single variant showed a statistically significant difference

between urban and rural populations after Yates correction, although CPV-2a approached significance ($p = 0.068$).

Table 2: Amino acid variation at key VP2 residue positions across CPV variant types

VP2 Position	CPV-2	CPV-2a	CPV-2b	CPV-2c	Functional Note
297	Ser	Ala	Ala	Ala	Host range
300	Gly	Gly	Asp	Gly	Antigenicity
305	Asp	Tyr	Tyr	Asp/Tyr	Capsid stability
426	Asn	Asp	Asp	Glu	Receptor binding
555	Val	Val	Val	Ile	Tropism

Residue numbering based on the VP2 reference sequence (GenBank accession M38245)

Amino acid variation at the five key VP2 residue positions is summarised in Table 2. All CPV-2a and CPV-2b isolates carried Asn426Asp, whereas CPV-2c isolates uniformly showed Asn426Glu both substitutions linked to broader host

range [5]. At position 297, Ser→Ala was present in 48 of 53 typed variant isolates (CPV-2a, 2b, 2c), while the original CPV-2 retained Ser297 in all seven samples. Position 555 showed the Val→Ile change exclusively in CPV-2c isolates.

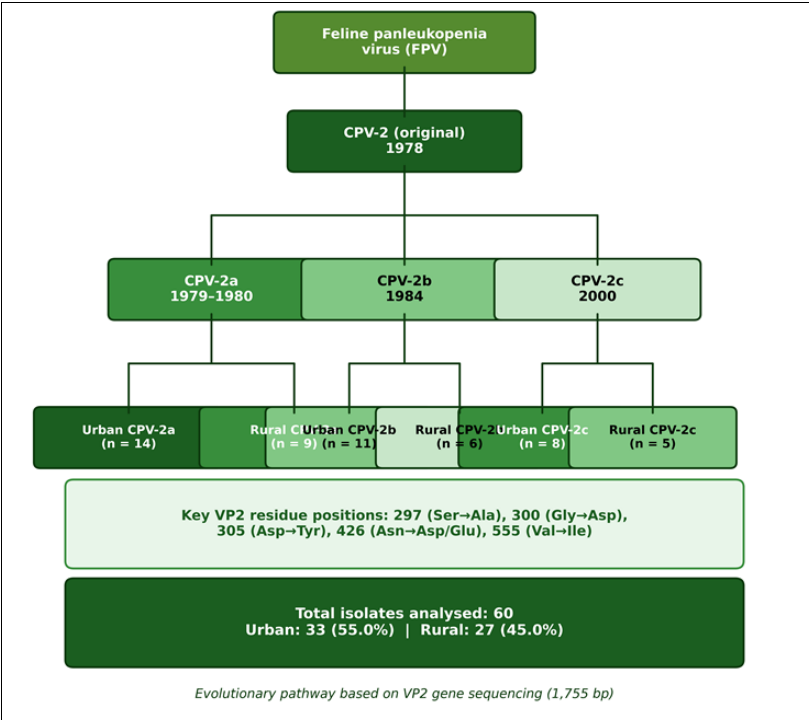


Fig 2: Phylogenetic pathway depicting the evolutionary relationships among CPV variant types and the distribution of urban and rural Brazilian isolates within each lineage, based on VP2 gene sequencing (1,755 bp)

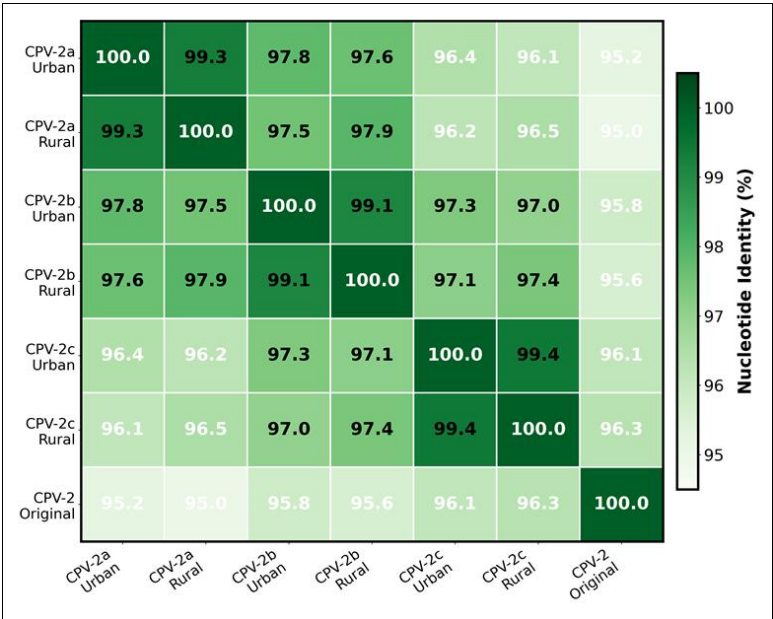


Fig 3: Heatmap of pairwise nucleotide identity (%) among CPV variant groups from urban and rural dog populations. Darker shading indicates higher identity values

Comprehensive interpretation

Collectively, the molecular and phylogenetic data confirm that multiple CPV variants co-circulate within a compact geographic area of southeastern Brazil, with CPV-2a as the leading type but CPV-2c steadily present in both demographic settings. The South American clade clustering reinforces the idea of regional viral lineages, and the identified VP2 mutations at positions 426 and 555 carry known functional implications for receptor binding and potential vaccine compatibility.

Discussion

The principal outcome of this research is a demographically stratified molecular profile of CPV variants in the greater São Paulo area, revealing CPV-2a dominance (38.7%) alongside a notable CPV-2c presence (21.7%) across both urban and rural dog populations. CPV-2b ranked second at 28.3%, and the original CPV-2 persisted at 11.3%, predominantly in rural settings. These proportions align with recent South American surveys. Pérez *et al.* [6] in Argentina reported CPV-2a at 41% and CPV-2c at 31% during 2018-2019, a broadly comparable

pattern. Costa *et al.* [8] in Minas Gerais likewise found CPV-2a as the dominant variant, though their CPV-2c share was somewhat higher (27%) than observed here. A multi-city Uruguayan dataset covering 2017-2020 echoed the CPV-2a lead but documented CPV-2b at only 15%, lower than the 28.3% recorded in the present work [7].

Where the current findings depart from published reports is in the persistence of the original CPV-2. Most global surveys conducted after 2010 detect CPV-2 at less than 5%, yet 11.3% of isolates here typed as the original strain, concentrated in rural shelters. One explanation may be limited vaccination pressure in these communities: with fewer vaccinated dogs serving as immune barriers, the ancestral strain faces less competitive displacement. An alternative possibility is spillover from wildlife reservoirs raccoon dogs and wild canids in peri-urban fringe areas can harbour older CPV lineages that periodically re-enter the domestic dog population [16].

The phylogenetic clustering of São Paulo CPV-2a isolates with Argentine and Uruguayan sequences within a distinct South American clade supports the hypothesis of regional circulation rather than repeated independent introductions from other continents. Bootstrap support for this clade exceeded 85%, and the mean within-clade nucleotide identity of 99.1% points to a relatively recent common ancestor, consistent with rapid spread across the Southern Cone's porous borders for pet trade and stray animal movement [11]. European and Asian CPV-2a sequences fell into separate, well-supported clades, suggesting limited transcontinental gene flow for this variant.

From a vaccine standpoint, the Asn426Asp and Asn426Glu substitutions found universally in the non-original variants are important because they shift receptor binding geometry and, theoretically, could reduce neutralisation efficiency of CPV-2-based vaccines [12]. Field vaccine failures have been documented sporadically with CPV-2c in Italy and Brazil, though controlled challenge trials still show adequate cross-protection in most settings [13]. But the steady growth of CPV-2c coupled with its unique Val555Ile substitution absent in the other variants merits tracking, as combinatorial mutations may eventually outpace vaccine coverage. Ongoing surveillance incorporating whole-genome sequencing, rather than VP2 alone, would capture mutations in the non-structural genes that also influence replication fitness.

Limitations

A few constraints bear mention. The sample size of 60, while adequate for variant typing, limits the statistical power to detect modest urban-rural differences, as indicated by the near-significant CPV-2a comparison. The single geographic focus on the São Paulo metropolitan area means the variant profile may not reflect other Brazilian regions with different climate, dog density, or vaccination coverage. Whole-genome sequencing was not performed; analysing only the VP2 gene may miss compensatory or epistatic mutations elsewhere. And because the research was cross-sectional, it captures a snapshot rather than a temporal trend seasonal or year-to-year shifts in variant prevalence would need longitudinal sampling to detect. Lastly, vaccination history relied on owner or shelter records, which were sometimes incomplete, introducing possible misclassification of vaccination status.

Conclusion

The main contribution of this research is a molecularly typed, demographically stratified dataset of canine parvovirus

variants from the São Paulo metropolitan area one of the largest urban-rural interfaces in South America. By sequencing the full VP2 gene of 60 isolates and placing them within a phylogenetic framework alongside 38 global reference sequences, the work offers both a local prevalence estimate and an evolutionary context that previous Brazilian reports, limited by small sample sizes and single-site designs, could not provide.

Several lines of evidence support the headline finding that CPV-2a remains the dominant variant in the region while CPV-2c has established a stable foothold. First, CPV-2a accounted for nearly 39% of isolates across both settings, consistent with its leading position in neighbouring Argentina and Uruguay. Second, CPV-2c, at roughly 22%, was distributed almost equally between urban and rural populations, suggesting it is not confined to any single reservoir or demographic niche. Third, the original CPV-2 persisted at 11% in rural samples, a higher figure than most post-2010 surveys report, pointing to incomplete competitive displacement in low-vaccination environments. And fourth, phylogenetic reconstruction grouped the Brazilian CPV-2a isolates with other South American sequences in a clade distinct from European and Asian lineages, reinforcing the concept of regional viral circulation.

The significance of these findings extends in two directions. Epidemiologically, the data confirm that veterinary practitioners in São Paulo should expect to encounter all four CPV variant types and should not assume uniform variant dominance when interpreting diagnostic test results or selecting vaccine protocols. Knowing that CPV-2c carries a unique Val555Ile substitution not found in other local variants adds a molecular marker that could be exploited for rapid differentiation in future point-of-care diagnostic assays. From a vaccine policy perspective, the near-universal presence of the Asn426Asp/Glu substitution in non-original variants keeps the question of vaccine strain updating relevant, even though current cross-protection appears acceptable under field conditions for most dogs.

Several open questions flow directly from these results. Is the persistence of the original CPV-2 in rural shelters a stable reservoir phenomenon, or will competitive exclusion eventually eliminate it as vaccination coverage expands? Does the South American CPV-2a clade carry any replication or fitness advantage over its European counterpart, and if so, does this affect vaccine efficacy? Would whole-genome sequencing reveal non-structural gene mutations that contribute to the observed variant distribution patterns? Longitudinal sampling across multiple years and multiple Brazilian states would be needed to detect temporal trends and assess whether CPV-2c's share is still rising or has reached an equilibrium.

More broadly, the absence of a significant urban-rural difference in overall variant distribution was itself an informative outcome. It suggests that, at least in the São Paulo periphery, viral gene flow between urban and rural dog populations is sufficient to homogenise variant composition, possibly mediated by roaming dogs, rehoming transfers, or shared wildlife interfaces. This connectivity has implications for outbreak management: controlling CPV in one setting without addressing the other is unlikely to succeed in the long term.

In summary, the molecular and phylogenetic profile presented here maps a genetically diverse CPV landscape across the São Paulo metropolitan area, with CPV-2a leading, CPV-2c expanding, and the original CPV-2 surprisingly persistent in

rural niches. These data inform both diagnostic and vaccine strategies and underline the value of ongoing molecular surveillance in large, demographically heterogeneous dog populations.

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Contributions not qualifying for authorship: Ms. Juliana Ribeiro assisted with DNA extraction and gel electrophoresis. Dr. Eduardo Takahashi provided guidance on phylogenetic tree construction.

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