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Identification of canine parvovirus with the Q370R point mutation in the VP2 gene from a giant panda (*Ailuropoda melanoleuca*)

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Abstract

Background: In this study, we sequenced and phylogenetic analyses of the VP2 genes from twelve canine parvovirus (CPV) strains obtained from eleven domestic dogs and a giant panda (*Ailuropoda melanoleuca*) in China. A novel canine parvovirus (CPV) was detected from the giant panda in China.

Results: Nucleotide and phylogenetic analysis of the capsid protein VP2 gene classified the CPV as a new CPV-2a type. Substitution of Gln for Arg at the conserved 370 residue in CPV presents an unusual variation in the new CPV-2a amino acid sequence of the giant panda and is further evidence for the continuing evolution of the virus.

Conclusions: These findings extend the knowledge on CPV molecular epidemiology of particular relevance to wild carnivores.

Keywords: Canine parvovirus, VP2 gene, Point mutation, Phylogenetic analysis, Giant panda

Background

Canine parvovirus type 2 (CPV-2) is an important pathogen in domestic dogs and several wild carnivore species. It was first identified in USA in 1978 and was found later to have spread worldwide in domestic and wild canine populations. CPV-2 is a small non-enveloped, single-stranded DNA virus (5.2 Kb) which is a member of the genus *Parvovirus* of the family *Parvoviridae*. The CPV genome is composed of two major ORFs, one encoding the two non-structural proteins [NS1 and NS2] and the other encoding the two capsid proteins [VP1 and VP2] [1]. There is also a third protein, VP3 which is produced by proteolytic processing of VP2.

CPV-2 is antigenically and genetically related to feline panleukopenia virus (FPLV). However, FPV infects cats, mink and raccoons, but not dogs, whereas CPV-2 infects dogs and other *Canidae*, but not cats. A few amino acid differences between CPV and FPV determine the specificity of these viruses [2]. After the CPV-2 initial appearance

(during 1978–1981), two new antigenic variants, named CPV-2a and CPV-2b, were characterized [3–5]. The antigenic types CPV-2a and CPV-2b differ from the original CPV-2 in at least five or six amino acids/residues of the VP2 capsid protein (genomic positions 3045, 3685, 3699, 4062 and 4449) [6,7]. Canine parvovirus type 2a/2b having mutation at 297 residue (Ser→Ala) is designated as new CPV-2a/2b [8,9], residue 297 is located in a minor antigenic site close to epitope B and substitutions at this position may be responsible for changes in antigenicity of CPV variants [10]. Another antigenic variant having an amino acid substitution 426-Asp→Glu was reported for the first time in Italy [11] and had been reported from other countries [1,12–17], and this variant is currently named as CPV-2c.

It has been reported that canine parvovirus (CPV) have been implicated in disease and mortality in giant pandas [18–21], which is an endangered species native to the China. The giant pandas with CPV infection showed diarrhea, vomiting and water-like feces [18]. Giant panda parvovirus VP2 gene described here identifies yet another variant of the virus. It demonstrates the continued adaptation of the virus to an everexpanding host range

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that includes endangered species of wildlife. Understanding emergent disease threats is important in enabling effective conservation measures for endangered species.

Results

Out of 36 faecal samples of giant pandas and 97 canine rectal swabs screened by PCR assay using Hfor/Hrev primers, 1 giant panda and 62 dog samples yielded a specific amplicon of 611 bp, respectively.

The amplified PCR products of 11 randomly selected canine samples and one giant panda sample were subjected for sequencing using primer pair Hfor/Hrev. Primer pair Hfor/Hrev [11] encompasses informative amino acid residues which are of significance in characterizing the CPV types. All the CPV samples under study were found to be new CPV-2a (CPV-2a with nucleotide variation T→G at position 3675 or CPV-2a with amino acid variation 297-Ser→Ala). In comparison to prototype new-CPV-2a (AY742953), the samples under this study had amino acid residue variations at Tyr324Ile caused by mutation TAT →ATT at nt 3756–3758 of the VP2 gene. It was a unique mutation within the VP2 of Chinese and Korean strains of “new CPV-2a”. Critical positions of the CPV VP2 gene products of samples sequenced in this study are summarized in Table 1.

In addition to the nucleotide variations at positions 3675 and 3756, three additional mutations were observed in the canine parvovirus sequences under study. One was at nucleotide position 3584 where a mutation (U→A) resulting in the codon change from UUC→UAC, with amino acid variation 267-Phe→Tyr. All the sequences under this study except B03, B06 and B11 showed this variation. The second one was at nucleotide position 4110, where variation A→G was observed and which changed the codon from ACG→GCG, with amino acid variation 442-Thr→Ala. This variation (A→G) at

nucleotide position 4110 was observed in strains A10, A11, A12, B01, B02, B05, B07 and B12 in this study (the dog samples). The last mutation was at nucleotide position 3894 where a mutation (A→G) resulting in the codon change from CAA→CGA, with amino acid variation 370-Gln→Arg. This variation only was revealed in strain B11 (the giant panda sample).

To analyse the phylogenetic relationships of the China isolates with other CPV strains isolated in various parts of the world, we constructed a maximum likelihood phylogenetic tree. The panda field isolate B11 was found to be phylogenetically closely related to new CPV-2a strains of Jilin strain CNJL0804. B06 and B03 are the closest of Chinese dog sequences examined in this study. Rest of the other sequences had distinct lineage but shared molecular relationship with new CPV-2a reference strains (Figure 1).

Discussion

New CPV-2a/2b appear to have replaced the prototype CPV-2a/2b strains and become the predominant types in many countries [22-26]. In China, the prevalent antigenic type in canine populations is type 2a, our results seem to confirm these data [27]. The dog and giant panda samples were all new CPV-2a, but it is impossible to conclude that new CPV-2a is predominant strain in the China dog or giant panda population because our data are limited to an exiguous number of samples and we do not have data regarding CPV-2 dog strains from the area where the giant panda samples were collected.

All the CPV clinical samples under study were found to be new CPV-2a (Figure 1). Similar substitution 297-Ser→Ala in CPV-2a strain were reported in VP2 gene of CPV from worldwide [2,22,28-30]. This study detected one site mutation among the CPV-2a isolates in China: Tyr→Ile at position 324, This mutation was also

Table 1 CPV strains from China, origin from which they were isolated and their GenBank accession numbers

Serial no	Strain	Host	Foster mode	Vaccinated	Origins	GenBank no.
1	A10	Dog	Captive	Yes	Rectal swabs	JX624761
2	A11	Dog	Captive	Yes	Rectal swabs	JX624762
3	A12	Dog	Captive	Yes	Rectal swabs	JX624763
4	B01	Dog	Captive	Yes	Rectal swabs	JX624764
5	B02	Dog	Captive	Yes	Rectal swabs	JX624765
6	B03	Dog	Captive	Yes	Rectal swabs	JX624766
7	B04	Dog	Captive	Yes	Rectal swabs	JX624767
8	B05	Dog	Captive	Yes	Rectal swabs	JX624768
9	B06	Dog	Captive	Yes	Rectal swabs	JX624769
10	B07	Dog	Captive	Yes	Rectal swabs	JX624770
11	B11	Giant panda	Captive	Yes	Faecal	JX624771
12	B12	Dog	Captive	Yes	Rectal swabs	JX624772

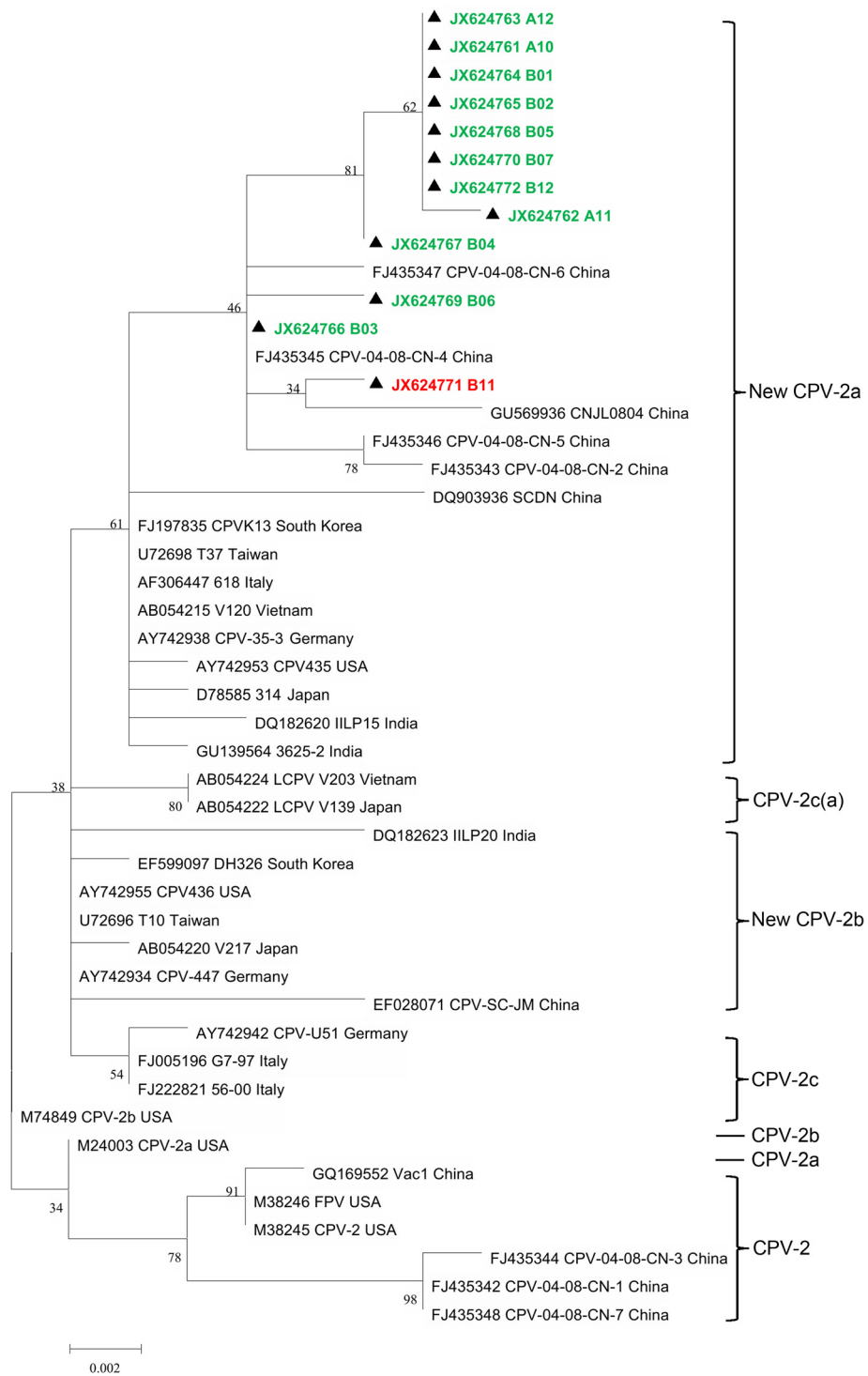


Figure 1 (See legend on next page.)

(See figure on previous page.)

Figure 1 Maximum likelihood tree (Mega-5.1 beta version) constructed using canine parvovirus sequences under study and the reference sequences.

M38246 (FPV, USA), M38245 (CPV-2, USA), M24003 (CPV-2a, USA), M74849 (CPV-2b, USA), AY742953 (New CPV-2a, USA), AY742955 (New CPV-2b, USA), DQ182620 (New CPV-2a, India), DQ182623 (New CPV-2b, India), D78585 (New CPV-2a, Japan), AB054220 (New CPV-2b, Japan), AY742938 (New CPV-2a, Germany), AY742934 (New CPV-2b, Germany), AY742942 (CPV-2c, Germany), AF306447 (New CPV-2a, Italy), AF306444 (CPV-2b, Italy), FJ222821 (CPV-2c, Italy), AB054222 (CPV-2c(a), Japan), AB054215 (New CPV-2a, Vietnam), AB054224 (CPV-2c(a), Vietnam), U72698 (New CPV-2a, Taiwan), U72696 (New CPV-2b, Taiwan), FJ197835 (New CPV-2a, South Korea), EF599097 (New CPV-2b, South Korea), DQ903936 (New CPV-2a, China), FJ435343 (New CPV-2a, China), FJ435342 (CPV-2, China), FJ435344 (CPV-2, China), FJ435345 (New CPV-2a, China), FJ435346 (New CPV-2a, China), FJ435347 (New CPV-2a, China), FJ435348 (CPV-2, China), EF599097 (New CPV-2b, South Korea), AB054215 (New CPV-2a, Japan), EF028071 (New CPV-2b, China), GQ169552 (CPV-2, China), Triangle (▲) indicates the 12 Chinese wild-type CPV strains analyzed in this study. Green and red were used for dog and giant panda strains, respectively.

reported in 2009 [31]. Previous studies have shown that residue 324 is subject to strong positive selection in all parvoviruses of carnivores [32].

The natural adaptation of a virus to a new host is a very rare event, suggesting that there are high barriers that prevent viruses from gaining the ability to infect and spread naturally in hosts to which they are not adapted. In this study, the novel point mutation virus is most likely not a panda-adapted virus spreading among pandas, but more likely a spill-over from dogs. Because host adaptation involved complex interactions among both surface-exposed and buried capsid mutations that together altered cell infection and immune escape properties of the viruses [33]. In this study, the virus VP2 gene coding change at nt 3894 (VP2 residue 370, Gln→Arg) was interesting because it has not been detected previously in any other strains. We speculated that the mutation has two aspect functions. On the one hand, residue 370 was close to residues 375 and 377, it has indicated that residues 375 and 377 are associated with the ability of CPV to hemagglutinate or alter pH dependence of hemagglutination [34], On the other hand, residue 370 was adjacent to residue 379 and 384, while the 379 and 384 residue affects canine transferrin receptor (TfR) binding to determined the canine host range [35]. So, we made the inferences that R370 might be involved in a required conformational change, or it might mediate an effect on receptor binding through the neighboring residues, and is likely to have had an effect on the parvovirus host range. This observation suggests that the glutamine to arginine mutation may also affect host DNA±protein interaction. As is the case for wild animal, this mutation is not selected for the population, but may have arisen independently from various backgrounds.

As the phylogenetic tree shows, most of the viruses isolated in China formed a large cluster, while some strains clustered together with viruses from regions outside China. Most of the CPVs isolated in China formed a specific cluster and certain mutations detected in Chinese CPVs probably arose during the process of local adaptation, as indicated by previous surveys [36].

Considering that the CPV-2 vaccine appears to provide a comparatively lower and shorter immunity against heterologous CPVs, there is evidences to suggest that complete immunity may not be provided to dogs even if CPV-2 vaccines are used [37-39]. In many countries, such as Europe, CPV-2a has been overtaken by CPV-2b or CPV-2c, some researches have been to evaluate antigenic relationships among the original canine parvovirus type 2 (CPV-2) and the variants CPV-2a, -2b, and -2c, cross-antigenic evaluation revealed clear differences among the CPV variants [40]. Nevertheless, our study showed that the giant panda and dogs are all new CPV-2a type, while the vaccines available for giant pandas and dogs are CPV-2 type vaccine in China (Nobivac®, Holland) [41]. The effectiveness of CPV-2 vaccine against CPV-2a type has not been evaluated in China.

In sum, wildlife in captive facilities in China is generally not reliably or safely vaccinated. Strain difference between field virus and vaccine candidate virus could be one of the important attributable reasons for some immunization failure. Infectious diseases pose a significant risk to these animals, of which many are endangered species, the mechanisms of the virus into the giant panda population and the adaptation (mutation) of the virus to that species are important topics for future research.

Materials and methods

Ethics statement

The animal from which specimens were collected, was handled in accordance with animal protection law of the People's Republic of China (a draft of an animal protection law in China released on September 18, 2009). This study was approved by the National Institute of Animal Health Animal Care and Use Committee at Sichuan Agricultural University (approval number 2010-020).

Clinical samples

A total of 36 faecal samples of giant pandas and 97 rectal swabs were collected from dogs suspected to be

Table 2 Alignment of the deduced amino acid sequences of partial VP2 gene

Aa residue	267	297	300	305	324	370	375	426	442
Nt position	3585-3588	3675-3577	3684-3686	3699-3701	3756-3769	3894-3897	3909-3912	4064-4067	4112-4115
M38245(CPV-2)	F	S	A	D	Y	Q	N	N	T
M24003(CPV-2a)	F	S	G	Y	Y	Q	D	N	T
M74849(CPV-2b)	F	S	G	Y	Y	Q	D	D	T
FJ222821(CPV-2c)	F	A	G	Y	Y	Q	D	E	T
AB054215(FPV)	F	A	G	Y	Y	Q	D	N	T
AY742953(New-CPV-2a)	F	A	G	Y	Y	Q	D	N	T
AY742955(New-CPV-2b)	F	A	G	Y	Y	Q	D	D	T
JX624761(A10)	Y	A	G	Y	I	Q	D	N	A
JX624762(A11)	Y	A	G	Y	I	Q	D	N	A
JX624763(A12)	Y	A	G	Y	I	Q	D	N	A
JX624764(B01)	Y	A	G	Y	I	Q	D	N	A
JX624765 (B02)	Y	A	G	Y	I	Q	D	N	A
JX624766(B03)	F	A	G	Y	I	Q	D	N	T
JX624767(B04)	Y	A	G	Y	I	Q	D	N	T
JX624768(B05)	Y	A	G	Y	I	Q	D	N	A
JX624769(B06)	F	A	G	Y	I	Q	D	N	T
JX624770(B07)	Y	A	G	Y	I	Q	D	N	A
JX624771(B11)	F	A	G	Y	I	R	D	N	T
JX624772(B12)	Y	A	G	Y	I	Q	D	N	A

Sequence of one of the representative canine parvovirus field isolate GU139553 (New CPV-2a) is shown aligned with the reference strains M38246 (FPV), M38245 (CPV-2), M24003 (CPV-2a), M74849 (CPV-2b), AY742953 (New CPV-2a), AY742955 (New CPV-2b) and FJ222821 (CPV-2c) obtained from the Genbank.

infected with CPV were gathered simultaneously. Detailed information on the origin and the accession numbers of the CPV-positive samples is shown in Table 2. All activities followed the legal requirements and institutional guidelines set out by the government of P.R. China. The samples were collected in China during a period of 7 months from November 2011 to May 2012. The collected samples were emulsified in 2 ml of 0.1 M PBS of pH 7.4 and centrifuged at 6000 g for 15 min at 4°C. The supernatant was collected and used for PCR amplification.

Template DNA preparation

Hundred microlitres of the processed supernatant was used for template DNA preparation by boiling at 96°C for 10 min and chilling immediately in crushed ice [42,43]. The supernatants were diluted 1:10 in distilled water to reduce residual inhibitors of DNA polymerase activity [12].

Primer pair and PCR amplification

PCR amplification was performed using KOD-Plus-Ver.2 (TOYOBO, Japan) and primer pair H_{for} (5'-CAGGTG ATGAATTTGCTACA-3')/H_{rev} (5'-CATTTGGATA - AACTGGTGGT-3') that amplifies 611 bp fragment of the gene encoding capsid protein [11]. PCR amplification

was consisted of 30 cycles of denaturation (95°C 45 s), annealing(51°C 45 s), extension (72°C 45 s) and final extension (72°C 10 min) and the products were analyzed by electrophoresis using 1.5% agarose gel in Tris acetate EDTA (TAE) buffer (1×).

Sequencing and phylogenetic analysis

PCR products of the correct size (611 bp in length) were amplified and cloned using TArget Clone-Plus-(TOYOBO, Japan), then custom sequenced with primer pair H_{for}/H_{rev}. The sequences were aligned with sequences of prototype CPV strains (M38246-FPV; M38245-CPV-2; M24003-CPV-2a; M74849-CPV-2b; AY742953- New CPV-2a; AY742955-New CPV-2b; FJ222821-CPV-2c) using Clustal W (<http://www.clustal.org>). The sequences were analyzed with respect to the prototype CPV-2 strain for the nucleotide variation of VP2 gene at positions 3675, 3684, 3699, 3756, 3909 and 4062 with the corresponding amino acid residues at 297, 300, 305, 324, 375 and 426, respectively.

For the phylogenetic analysis, 35 canine parvovirus sequences from various parts of the world were retrieved from the GenBank and used. The sequences were aligned using Clustal W. Maximum likelihood tree was drawn using the MEGA 5.0 Software [44].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: LG QGY. Performed the experiments: LG SLY ZXH MLZ ZFQ. Analyzed the data: LG SLY MLZ ZXH. Contributed reagents/materials/analysis tools: SJC ZHZ RH YPR CDW XTW SJC WZG. Wrote the paper: LG SLY. All of the authors read and approved the final version of the manuscript.

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