Evaluation of the Antigenic Relationships among Canine Parvovirus Type 2 Variants

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The antigenic relationships among the original canine parvovirus type 2 (CPV-2) and the variants CPV-2a, -2b, and -2c were evaluated. Cross-antigenic evaluation revealed clear differences among the CPV variants, which were more appreciable by serum neutralization (SN) than by hemagglutination inhibition. Antigenic differences were found mostly between the original CPV-2 and the variants, but they were also observed among the variants CPV-2a, -2b, and -2c. The variant CPV-2c exhibited a unique antigenic pattern, since it was poorly recognized by the sera of animals immunized with CPV-2, CPV-2a, and CPV-2b. However, animals immunized with CPV-2c exhibited higher SN titers to CPV-2b than to the homologous virus CPV-2c. The observed antigenic differences might drive selection of CPV strains by generating differential immune pressure in the canine population, which raises concerns about vaccine efficacy.

Canine parvovirus type 2 (CPV-2) is responsible for a severe, highly contagious gastrointestinal disease in pups. CPV-2 was first identified in the late 1970s, when outbreaks of fatal myocarditis and hemorrhagic gastroenteritis were observed in young puppies worldwide (3, 8, 23, 24). By sequence analysis CPV-2 appeared to be closely related to feline parvovirus (FPV) and also to parvoviruses from raccoons, minks, and artic foxes (30, 41), with the nucleotide variation from FPV being lower than 0.5%. In the 1980s the original CPV-2 was completely replaced by new antigenic variants designated CPV-2a and CPV-2b, and the original virus is no longer present in the canine population and exists only in the vaccine formulations. There are at least six or seven amino acid changes between FPV and CPV-2 and at least five or six amino acid changes between the variants CPV-2a/b and the original CPV-2 in the VP2 capsid protein (31, 32), while the variant CPV-2a differs from the variant CPV-2b only in the change 426-Asn→Asp within the major antigenic site of the capsid (Table 1) (31, 32). Soon after the appearance of the CPV-2a/b variants, a number of additional, unusual mutations affecting important residues of the capsid protein VP2 of CPV were recognized (Table 1), suggesting that CPV-2 is still evolving (6, 22, 42). One such variant, Glu-426 (CPV-2c) appears to be widespread in Europe (15, 25) and has been detected in the Asiatic and American continents as well (20, 28, 34).

The few amino acids differences in FPV, CPV-2, and CPV-2a/b appear to have altered the antigenic features of the virus and to have modified important biological properties, such as the in vivo and vitro host ranges (36, 43, 44), the interactions with the cellular receptor, the transferrin protein (21, 29), and the virulence (9). Also, there is concern that the vaccines used currently to prevent CPV infection in dogs may fail to effectivELY protect pups against the new CPV antigenic variants (40). Although the original CPV-2 was completely replaced by the antigenic variants a few years after its appearance, the original CPV-2 is still used in most commercial vaccines. Several studies have demonstrated that CPV-2 vaccines are still effective to induce protection against CPV variants (9, 18, 39, 45). However, new modified live (ML) vaccines have been developed and licensed using CPV-2b strains.

Studies with antisera raised against the original CPV-2 and the variants have been performed to test the amount of neutralizing activity, particularly against the heterologous types. These studies have revealed substantial difference in the neutralization titers and have suggested that the hemagglutination (HA)-inhibiting antibodies do not correlate well with the neutralizing antibodies and may incorrectly estimate the protective immunity against the antigenic variants in pups with passively acquired antibodies against the original type of CPV (37, 40). In this study, the antigenic relationships among the original CPV-2 and the variants CPV-2a, -2b, and -2c were evaluated by HA inhibition (HI) and serum neutralization (SN) in order to acquire more conclusive data on the antigenic relationships among the various CPV-2 variants.

MATERIALS AND METHODS

Cells. Virus cultivation and SN were performed on the canine A-72 cell line grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Viruses. Four CPV-2 strains were used in the study. Strain 17/80 ISS, with a titer of 3.2×10⁵ 50% tissue culture infectious doses (TCID₅₀/50 ml), was used as a representative of the original CPV-2 (5). Strain 192/98 (3.2×10⁵ TCID₅₀/50 ml) was used as representative of the CPV-2a variant. The virus was obtained from the feces of a pup that died from CPV-induced gastroenteritis in 1998 in Bari, Italy. Strain 29/97 (3.2×10⁶ TCID₅₀/50 ml) (4–7) and strain 136/00 (3.2×10⁶ TCID₅₀/50 ml) (6) were used as representatives of CPV variants 2b and 2c, respectively. Titration of the viral strains was performed in microtiter plates. Tenfold virus dilutions were prepared in quadruplicates in DMEM and were added to wells with 2×10⁴ A-72 cells/well. After incubation at 37°C for 4 days in a CO₂ atmosphere, the plates were frozen and thawed three times, and the undiluted cytopathic of each well was tested by HA using 1% pig erythrocytes. The virus titer was considered the end point dilution showing HA activity in 50%
prepared in normal adult rabbits. The antigen for rabbit hyperimmunization was
obtained from eight dogs inoculated subcutaneously with 1 ml of undiluted
CPV-2. Rabbit sera. Antisera against CPV-2, CPV-2a, CPV-2b, and CPV-2c were
produced in normal adult rabbits. The antigen for rabbit hyperimmunization was
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TABLE 2. Antibody titers in canine sera as measured by HI and SN tests 30 days (T1) after vaccination or infection with CPV-2 or its antigenic variants

| Dog group (virus) | Antibody raised | Antibody titera | P valueb | Antibody titera | P valueb |
|-------------------|-----------------|-----------------|----------|-----------------|----------|
|                   |                 | HI              |          |                 |          |
|                   |                 | Least-square mean | Geometric mean |          | Least-square mean | Geometric mean |
| A (CPV-2)         | CPV-2           | 11.82 ± 0.39    | 3,620    |                 |          |
|                   | CPV-2a          | 10.82 ± 0.39    | 1,810    |                 |          |
|                   | CPV-2b          | 10.48 ± 0.37    | 1,234    | 0.019***        | 8.44 ± 0.45 | 354 | <0.001*** |
|                   | CPV-2c          | 10.32 ± 0.42    | 1,395    | 0.014***        | 9.54 ± 0.42 | 842 | <0.001*** |
| B (CPV-2b)        | CPV-2           | 10.21 ± 0.22    | 1,185    | <0.001***      | 10.76 ± 0.54 | 1,741 | NS |
|                   | CPV-2a          | 11.32 ± 0.22    | 2,593    | NS              | 9.60 ± 0.53 | 766  | NS |
|                   | CPV-2b          | 11.37 ± 0.21    | 2,677    |                 | 11.02 ± 0.52 | 2,282 | NS |
|                   | CPV-2c          | 11.20 ± 0.23    | 2,370    | NS              | 9.38 ± 0.38 | 723  | 0.042a |
| C (CPV-2c)        | CPV-2           | 11.57 ± 0.34    | 3,044    | NS              | 13.82 ± 0.42 | 14,481 | <0.001*** |
|                   | CPV-2a          | 12.20 ± 0.35    | 4,764    | NS              | 10.57 ± 0.43 | 1,522 | NS |
|                   | CPV-2b          | 11.52 ± 0.31    | 2,560    | NS              | 11.92 ± 0.39 | 5,120 | 0.026a |
|                   | CPV-2c          | 11.32 ± 0.40    | 3,044    |                 | 10.32 ± 0.50 | 1,280 |          |

a Homologous values are in boldface.

b The statistical significance of the comparison between homologous and heterologous titers is rated as follows: NS, not significant; *, significant; ** or ***, highly significant.

18,780, whereas the heterologous SN titers were 354, 842, and 348 for CPV-2a, CPV-2b, and CPV-2c, respectively. All the differences were statistically significant (P < 0.001).

In dogs immunized with CPV-2b (group B), the homologous HI titer (geometric mean) was 2,677 and the heterologous HI titers were 1,185, 2,593, and 2,370 for CPV-2, CPV-2a, and CPV-2c, respectively, with a significant difference against CPV-2 (P < 0.001). By SN, the homologous titer was 2,282 and the heterologous titers were 1,741, 766, and 723 for CPV-2, CPV-2a, and CPV-2c, respectively. There was a statistically significant difference (P = 0.042) in the heterologous titer against CPV-2c.

In dogs naturally infected by CPV-2c (group C) there was no statistically significant difference between the homologous HI titer (3,044) and the heterologous titers against CPV-2 (3,044), CPV-2a (4,764), and CPV-2b (2,560). By SN, the homologous titer was 1,280 and the heterologous titers against CPV-2, CPV-2a, and CPV-2b were 14,481, 1,522, and 5,120, respectively. Statistically significant differences were observed against CPV-2 (P < 0.001) and CPV-2b (P = 0.026).

Rabbit sera. The last-square and geometric means of the HI and SN titers against the four CPV variants in the T1 (30 days after immunization) and T2 (80 days after immunization) sera are reported in Table 3 and Table 4, respectively.

In the sera from the rabbits inoculated with CPV-2c (D1 and D2), the only statistically significant difference in the T1 and T2 HI titers was found against the original CPV-2. Intriguingly, by SN the T1 and T2 titers against the homologous virus were significantly lower than the titers against the variant CPV-2b. Differences were also observed in the T2 titer against the original type.

DISCUSSION

The antigenic relationships among the original CPV-2 and the variants CPV-2a, CPV-2b, and CPV-2c were evaluated by HI and SN using the sera of immune dogs and rabbits. Inoculation of rabbits with the various CPV-2 strains was done in order to obtain a monospecific serological response, as rabbits, unlike dogs, are not a natural host of CPV-2 infection and therefore may not experience previous “priming” by CPV. Cross-antigenic evaluation of the CPV-2 variants revealed clear differences, which were more appreciable by SN than by HI. These findings confirm preliminary observations (37) and deserve particular attention, as HI is the gold standard test used in diagnostic laboratories for evaluation of humoral immunity to CPV-2. Accordingly, the results obtained with HI may tend to overrate the real immune status of the animals.

As previously observed (37), the greatest antigenic differences were found between the original CPV-2, which is still largely employed in vaccine formulations, and the variants. This finding was not unexpected, since the original CPV-2 differs in at least five or six amino acid changes from the recent CPV-2 variants (31). However, it was also possible to observe antigenic differences among the CPV-2a, CPV-2b, and CPV-2c variants, which may differ from each other even by a single
Amino acid change (27). In the animals immunized with CPV-2, the SN titers to the antigenic variants CPV-2a, CPV-2b, and CPV-2c were significantly lower than the homologous titers (raised to the original type). It is improbable that these differences may account for decreased protection against the variants in dogs that are protected by a strong active immune response. However, it is possible that these differences may allow escape from the limited antibody repertoire of maternal origin in young, unvaccinated pups. Severe parvovirus outbreaks have been observed in pups with HI titers of maternally derived antibodies above the threshold (1:80) related to protection against disease and infection (C. Buonavoglia, unpublished data). Likewise, experimental infection by virulent

| Rabbits (virus) | Antibody raised | HI | SN |
|----------------|----------------|----|----|
|                 | Antibody titera | P valueb | Antibody titera | P valueb |
|                 | Least-square mean | Geometric mean |             | Least-square mean | Geometric mean |
| A1A2 (CPV-2)    | CPV-2           | 8.82 ± 0.50 | 452 | 10.82 ± 0.40 | 1,810 |
|                 | CPV-2a          | 7.82 ± 0.50 | 226 | 7.82 ± 0.40 | 226 | 0.003** |
|                 | CPV-2b          | 7.82 ± 0.50 | 226 | 8.82 ± 0.40 | 452 | 0.022** |
|                 | CPV-2c          | 7.82 ± 0.50 | 226 | 7.82 ± 0.40 | 226 | 0.003** |
| B1B2 (CPV-2a)   | CPV-2           | 8.32 ± 0.47 | 320 | 0.053* | 10.32 ± 0.13 | 1,280 |
|                 | CPV-2a          | 9.82 ± 0.47 | 905 | 10.32 ± 0.13 | 1,280 |
|                 | CPV-2b          | 9.82 ± 0.47 | 905 | 11.07 ± 0.13 | 2,217 | 0.003** |
|                 | CPV-2c          | 9.82 ± 0.47 | 905 | 9.32 ± 0.13 | 640 | <0.001*** |
| C1C2 (CPV-2b)   | CPV-2           | 7.82 ± 0.47 | 226 | 8.82 ± 0.57 | 452 | <0.001*** |
|                 | CPV-2a          | 10.82 ± 0.47 | 1,810 | NS | 10.82 ± 0.57 | 1,810 | 0.017* |
|                 | CPV-2b          | 10.32 ± 0.47 | 1,357 | 12.82 ± 0.57 | 7,240 |
|                 | CPV-2c          | 9.82 ± 0.47 | 905 | 8.82 ± 0.57 | 452 | <0.001*** |
| D1D2 (CPV-2c)   | CPV-2           | 9.82 ± 0.35 | 905 | 0.004** | 11.32 ± 0.10 | 2,560 |
|                 | CPV-2a          | 10.82 ± 0.35 | 1,810 | NS | 10.32 ± 0.10 | 1,280 | <0.001*** |
|                 | CPV-2b          | 11.82 ± 0.35 | 6,202 | NS | 14.32 ± 0.10 | 20,480 | <0.001*** |
|                 | CPV-2c          | 11.82 ± 0.35 | 3,620 | 11.32 ± 0.10 | 2,560 |

*a* Homologous values are in boldface.

*b* The statistical significance of the comparison between homologous and heterologous titers is rated as follows: NS, not significant; *, significant; ** or *** highly significant.

| Rabbits (virus) | Antibody raised | HI | SN |
|----------------|----------------|----|----|
|                 | Antibody titera | P valueb | Antibody titera | P valueb |
|                 | Least-square mean | Geometric mean |             | Least-square mean | Geometric mean |
| A1A2 (CPV-2)    | CPV-2           | 9.82 ± 0.50 | 905 | 11.82 ± 0.40 | 3,620 |
|                 | CPV-2a          | 8.82 ± 0.50 | 452 | 8.82 ± 0.40 | 452 | 0.003** |
|                 | CPV-2b          | 7.82 ± 0.50 | 226 | 10.82 ± 0.40 | 1,810 | 0.004** |
|                 | CPV-2c          | 8.82 ± 0.50 | 452 | 9.32 ± 0.40 | 905 | 0.022* |
| B1B2 (CPV-2a)   | CPV-2           | 9.32 ± 0.47 | 640 | NS | 11.32 ± 0.13 | 2,560 | 0.003** |
|                 | CPV-2a          | 11.32 ± 0.47 | 2,560 | NS | 12.07 ± 0.13 | 4,434 |
|                 | CPV-2b          | 10.32 ± 0.47 | 1,280 | NS | 12.32 ± 0.13 | 5,120 | NS |
|                 | CPV-2c          | 11.32 ± 0.47 | 2,560 | 9.32 ± 0.13 | 640 | <0.001*** |
| C1C2 (CPV-2b)   | CPV-2           | 8.82 ± 0.47 | 452 | <0.017* | 10.82 ± 0.57 | 1,810 | <0.001*** |
|                 | CPV-2a          | 11.82 ± 0.47 | 3,620 | NS | 12.82 ± 0.57 | 3,620 | 0.005** |
|                 | CPV-2b          | 10.82 ± 0.47 | 2,715 | NS | 14.32 ± 0.57 | 20,480 |
|                 | CPV-2c          | 10.82 ± 0.47 | 1,810 | NS | 9.82 ± 0.57 | 905 | <0.001*** |
| D1D2 (CPV-2c)   | CPV-2           | 11.32 ± 0.35 | 2,560 | 0.004** | 13.32 ± 0.10 | 10,240 | <0.001*** |
|                 | CPV-2a          | 12.32 ± 0.35 | 5,120 | NS | 12.32 ± 0.10 | 5,120 |
|                 | CPV-2b          | 13.32 ± 0.35 | 10,240 | NS | 14.32 ± 0.10 | 20,480 | <0.001*** |
|                 | CPV-2c          | 13.32 ± 0.35 | 10,240 | 12.32 ± 0.10 | 5,120 |

*a* Homologous values are in boldface.

*b* The statistical significance of the comparison between homologous and heterologous titers is rated as follows: NS, not significant; *, significant; ** or *** highly significant.
CPV-2b strains of unvaccinated pups with high maternally derived antibody HI titers (≥80), which are usually expected to prevent CPV infection and disease, resulted in clinical signs, virus shedding, and an antibody response (14, 16).

Although animals immunized correctly with CPV-2 vaccines are fully protected clinically (2, 18), there is evidence that the active immunity elicited by the vaccines may sometimes fail to protect adult dogs, and the reasons for this may rely on a physiological decline of the protective immunity or on the increased virulence/tropism inherent to some CPV strains. Infection of adult dogs by CPV-2 is uncommon, as CPV-2 usually causes enteritis in young pups (1, 35). However, sporadic cases of CPV-2c infection in adult dogs (>1 year) have been diagnosed in our laboratories (6, 10; Buonavoglia, unpublished data). More recently, we observed a large outbreak of disease caused by CPV-2c in adult dogs housed in a breeding kennel. All the dogs had been immunized three times with a vaccine containing the original CPV-2, followed by a yearly booster vaccination (12). In this case, decreased levels of immunity in the adult dogs, coupled with mechanisms of antigenic escape and/or modified age-related tropism by the CPV-2c variant, are possible reasons that may have contributed to facilitate the virus spread and the onset of the disease in this animal group. These findings raise doubts about the real duration and level of immunity induced by CPV-2 vaccines in dogs, notably in view of the new guidelines for vaccine prophylaxis in dogs, which suggest booster vaccinations at 3-year intervals (33).

Interestingly, it was also possible to observe differences among the antigenic variants CPV-2a, CPV-2b, and CPV-2c. Based on the fact that the original CPV-2 does not exist any longer in the field and on the proposition that the antigenic differences may somehow decrease the effectiveness of the vaccines (40), new ML vaccines using CPV-2b strains have been developed and licensed. In our study, marked antigenic differences were observed by SN in the sera of dogs and rabbits immunized with the CPV-2b vaccine, as the heterologous SN titers (versus CPV-2a and -2c) were significantly lower than the homologous SN titer (versus CPV-2b).

Even more interestingly, evaluation of the antigenic features of CPV-2c by cross-neutralization revealed a unique pattern for the variant CPV-2c. This variant was first identified in 2000 in Italy and became predominant in a few years (25, 27). Subsequently, it has been identified in other European countries and in the Asiatic and American continents (11, 28, 20, 34). The spread of such a CPV-2 mutant may be accounted for by changes in biological properties, such as improved adaptation to the canine host and/or stabilization of the capsid structure, or by mechanisms of antigenic escape triggered by the change Asn/Asp-426→Glu (26). In this study, the CPV-2c variant was less effectively recognized in SN by the sera of dogs and rabbits inoculated with the heterologous (CPV-2, -2a, and -2b) viruses. Conversely, in dogs and rabbits infected/inoculated with CPV-2c, the homologous (versus CPV-2c) titers tended to be lower than the heterologous titers, notably versus CPV-2b. To a lesser extent, a similar inconsistent pattern was observed in rabbits inoculated with the variant CPV-2a, as the homologous (versus CPV-2a) titers tended to be lower than the heterologous titers to CPV-2b. A similar antigenic paradox has been observed by analysis of porcine parvovirus (PPV) strains. By SN using immune porcine and rabbit sera, the highly virulent strain PPV 27a displayed homologous titers 100 to 1,000-fold lower than the heterologous titers raised against other PPV strains (46).

That the antigenic paradox exhibited by CPV-2c may generate a different selective pressure in the dog population and may have contributed to the spread of the variant CPV-2c is an intriguing hypothesis. Also, these findings warrant studies to evaluate the opportunity to develop ML vaccines based on the CPV-2c variant.

In conclusion, the findings of this study indicate discrepancies between the HI and SN titers, suggesting that HI is not adequate to evaluate the real protective immunity of dogs, in particular against the antigenic variants. Also, by SN we observed significant differences in the homologous and heterologous antibody titers. These differences were more marked between the original CPV-2 and the recent variants CPV-2a, CPV-2b, and CPV-2c. However, significant differences were also observed among the CPV-2 variants. Like the human influenza virus and human rotavirus vaccines (17, 19), viruses containing strains matching the antigenic features of the field strains circulating in the local canine population, or polyvalent vaccines, could represent an alternative strategy to improve the effectiveness of prophylaxis for CPV-2.

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