Detection by PCR of Wild-Type Canine Parvovirus Which Contaminates Dog Vaccines

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A method for detecting wild-type canine parvovirus (CPV) strains which contaminate vaccines for dogs has been developed by PCR. PCR primers which distinguish vaccine strains from the most common, recent strains of wild-type CPV in many countries, including Japan and the United States, were developed. This PCR is based on the differences in nucleotide sequences which determine the two antigenic types of this virus. CPV vaccine strains derived from antigenically old-type virus prevalent in former times were not detected by PCR with differential primers. Detection sensitivity of PCR was 100- to 10,000-fold higher than that of the culture method in Crandell feline kidney cells.

Most of the important viral diseases of dogs can be well controlled by vaccines. Many kinds of vaccines are available, and most contain combinations of viruses and other antigens. The quality control of vaccines is a significant problem. In order to supply safe and efficient vaccines, each batch or lot of vaccine is inspected carefully through many tests before release. Of all these tests, special care is given to tests for safety. The test for extraneous viruses which contaminate vaccines is one of the most important tests for live-virus vaccines. Some viral vaccines are prepared in primary cell cultures, in which some extraneous virus could lurk. Even a cell line culture could be contaminated with viruses which show no or very weak cytopathogenic effect. Some viruses which are extremely stable in such an environment could contaminate the hands, clothes, or shoes of persons and be carried into vaccine preparations. If some extraneous virus contaminated a live-virus vaccine through any process of vaccine preparation, the contaminant virus could remain active until the vaccine is given to animals. The importance of tests for extraneous viruses has been realized through a number of episodes in which vaccines were contaminated, for example, simian virus 40 in the poliovirus vaccine (17) and reticuloendotheliosis virus in the Marek's disease vaccine (19).

The present test to detect extraneous virus in live-virus vaccines is performed after the neutralization of viruses in the vaccine by relevant antisera, whereby antiserum neutralizes the vaccine strain as well as the wild-type strain of the same virus species. Samples are then inoculated into cultured cells. In this system, if the vaccine contains several important viruses, any contaminating wild-type strains of the same species are not detected.

PCR (15) is characterized by high sensitivity, specificity, and rapidity and has become widely used for the detection of many different microorganisms (4, 9, 10). Therefore, we have tested here the potential for detecting extraneous viruses by PCR, with canine parvovirus (CPV) as the model.

Each vaccine strain should have some specific markers to distinguish it from wild-type strains. Most markers are biological characters, though the genetic bases of these markers are often not defined. It is necessary for differential detection of wild-type virus by PCR that the genetic markers of vaccine strains have been clarified.

CPV is a recently emerged pathogen of dogs; it was first observed in 1978 (1, 3). We have previously shown the strain replacement of CPV from the original virus (designated CPV type 2) to variants (designated CPV type 2a and CPV type 2b) (13, 14, 16). The new types of this virus emerged around 1979 and during the mid-1980s, respectively, and have become more dominant yearly. At present, almost all live-virus vaccine strains are the original CPV type 2 strain, as they were developed immediately after the emergence of CPV, and therefore have natural markers of CPV type 2 antigenicity and sequence type. We recently determined the differences in nucleotide sequence between the old- and new-type strains (12); therefore, we present here a detection procedure for wild-type CPV type 2a and type 2b strains in vaccines from CPV type 2-derived vaccine strains by PCR on the basis of those sequence differences.

MATERIALS AND METHODS

Virus strains. A total of 162 CPV isolates collected from various areas in Japan and the United States between 1979 and 1992 were divided into antigenic types with monoclonal antibodies as described previously (16). Fifteen of these isolates were tested by PCR (Table 1). All of the other type 2a and 2b strains from Japan were also tested by PCR to confirm that the sequences of the primers were conserved among type 2a and 2b strains.

The TU1 strain of feline panleukopenia virus (8), the Abashiri strain of mink enteritis virus (6), the 90HS strain of porcine parvovirus (11), the Nakano strain of canine distemper virus (7), the A2 strain of canine adenovirus type 2 (18), the T1 strain of canine parainfluenza virus (2), the CVS strain of rabies virus, and the D43 strain of canine infectious hepatitis virus were also used to test the specificity of primers for related viruses and other dog viruses.

Vaccines. All four kinds of live-virus vaccines which include CPV vaccine strains and are commercially available in Japan were used. There are two monovalent CPV vaccines (A is liquid and B is lyophilized) and two combined vaccines (C and D are lyophilized and contain canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, and CPV).

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TABLE 1. Strains subjected to PCR

Type and strain no.	Code or designation	Yr of isolation
Old (2)		
1	b[a]	1978
2	Cp49	1979
3	29-F	1980
4	Towada 2	1980
5	Cp82031	1982
New (2a)		
6	CP-K1	1979
7	Kawazoe	1983
8	15[a]	1984
9	TDKeT9142	1991
10	AQN9264	1992
New (2b)		
11	Y-1	1982
12	Cp83016	1983
13	39[a]	1984
14	TDKeT87197	1987
15	4644	1990

[a] Isolated in the United States.

TABLE 2. Antigenic types of CPV isolates collected from 1978 to 1992 in Japan and the United States

Yr of isolation	No. of isolates			
	Japan		United States	
	Old type	New type (2a/2b)	Old type	New type (2a/2b)
1978	0	0 (0/0)	5	0 (0/0)
1979	3	1 (1/0)	9	1 (1/0)
1980	6	1 (1/0)	6	5 (5/0)
1981	2	1 (1/0)	2	11 (11/0)
1982	1	5 (4/1)	0	2 (2/0)
1983	0	3 (1/2)	0	7 (7/0)
1984	0	6 (1/5)	0	8 (6/2)
1985	0	1 (0/1)	1	3 (3/0)
1986	0	1 (0/1)	0	3 (3/0)
1987	0	1 (0/1)	0	1 (0/1)
1988	—[a]	—	2	12 (2/10)
1989	0	3 (2/1)	0	2 (0/2)
1990	0	7 (6/1)	0	12 (2/10)
1991	0	13 (13/0)	—	—
1992	0	15 (15/0)	—	—

[a] —, samples were not collected.

Differential primers for PCR. From the sequences of CPV type 2, type 2a, and type 2b (12), we designed differential primers for the new-type strains as shown in Fig. 1. CPV type 2a or 2b nucleotides which differed from the sequences of CPV type 2 were included as the 3′ termination of each primer.

PCR. PCR was performed with a Program Temp Control System PC-700 (Astec, Fukuoka, Japan). Samples which contained virus as templates were directly added to PCR mixtures without the extraction of DNA. The conditions for PCR have been described previously (5). Briefly, amplification was achieved by 30 cycles of denaturation at 94°C for 30 s, renaturation at 55°C for 2 min, and elongation at 72°C for 2 min. PCR products were electrophoresed on 1% agarose (SeaKem GTG) gels; gels were stained with ethidium bromide and then visualized with UV light.

Detection sensitivity of PCR. The TDKeT9142 isolate was used as the prototype of CPV type 2a, and the Y-1 isolate was used as the prototype of CPV type 2b. Virus samples which contained a 50% tissue culture infectious dose ($TCID_{50}$) per µl were diluted 10-fold serially with each vaccine or phosphate-buffered saline (PBS). One microliter of each dilution was used in PCR. The detection sensitivities of PCR were compared with the $TCID_{50}$ of the same virus stock in Crandell feline kidney cells.

Application of the test for extraneous virus in vaccines. A total of 40 batches of all four kinds of vaccines which include CPV vaccine strains were tested for extraneous virus by PCR.

RESULTS

Typing with monoclonal antibodies and differentiation by PCR. The results of antigenic typing of 162 isolates from Japan and the United States with monoclonal antibodies are shown in Table 2. The isolation rates of new (2a and 2b)-type strains increased yearly from 1979 to 1982, and all 50 isolates collected in Japan and 48 of 51 isolates in the United States after 1983 were new-type strains. Five isolates of each antigenic type (2,

2a, and 2b [Table 2]) were randomly selected and tested by PCR. Agarose gel electrophoretic patterns of PCR products from these 15 isolates are shown in Fig. 2. There were no detectable DNA bands from CPV type 2 isolates, but clear bands of the expected sizes were obtained from all CPV type 2a and 2b isolates. PCR with all of the other type 2a and 2b strains from Japan also resulted in amplification of the same-size DNA (data not shown).

Detection sensitivity of PCR. PCR products obtained after serial 10-fold dilution of new-type (2a and 2b) strains in PBS are shown in Fig. 3. The detection limits of PCR were 10^{-3} to 10^{-4} $TCID_{50}$ in 1 µl of sample. PCR products obtained after serial 10-fold dilution of the CPV type 2a and 2b strains in four different kinds of vaccine which contain CPV type 2 vaccine strains are shown in Fig. 4. While the detection limits of type 2a and type 2b CPV in monovalent CPV vaccines were comparable to those obtained after dilution in PBS, the detection of new virus sequences when diluted in combined vaccines was 1/10 to 1/100 of that obtained after dilution in PBS.

Specificity of primer. Feline panleukopenia virus and mink enteritis virus (which belong to the same virus group), porcine parvovirus (which belongs to the same genus), and several other unrelated dog viruses (canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, rabies virus, and canine hepatitis virus) were added to PCR mixtures with the


```
                          Genome of CPV
  5´                                                    3´
  0      1000     2000     3000     4000     5000
  |--------------------------------------------|
                         ▲        ▲
                        VP1      VP2        Antisense primer RY1
            Sense primer FY3

                                          3´ CATGATTGAAACCAATATATCC
         5´ GAAGAGTGGTTGTAAATAATT      GTACTAACTTTGGTTATATAGG
            3025              3045    3685         3699   3706
  CPV-2  (old) --------------------A   C------------G-------
   2a   (new)  --------------------T   G------------T-------
   2b   (new)  --------------------T   G------------T-------
```
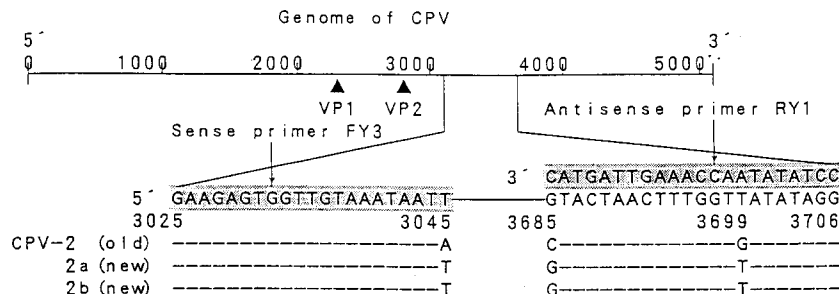
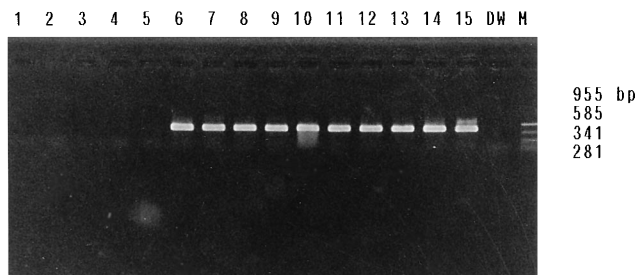FIG. 1. Differential primers for new-type CPV (CPV type 2a and CPV type 2b).

FIG. 2. Differentiation of antigenic type by PCR with differential primers. Lanes 1 to 15, agarose gel electrophoretic pattern of PCR products from strains 1 to 15 (Table 1), respectively. Lane DW, distilled water (negative control); lane M, molecular mass markers.

same primers, but no detectable DNA bands were produced by PCR (data not shown).

Results of the test for extraneous virus in vaccines by PCR. No DNA amplification was observed in the test for extraneous virus by PCR with vaccines currently in use (data not shown).

DISCUSSION

The tests for extraneous viruses regulated by the minimum requirements for biological products for veterinary use in Japan are divided into two types. One is for specified viruses, such as hog cholera virus in porcine vaccines or reticuloendotheliosis virus in poultry vaccines. Other tests are for unspecified viruses in all live-virus vaccines. Both tests employ culture methods in suitable cells, eggs, or suckling mice. The viruses of vaccine strains are neutralized by relevant antisera before culture. In the nonspecific assay for viruses, it is likely that some wild-type viruses will not grow directly in the limited kinds of cell cultures presently employed. It takes much time and labor for the test itself and for preparation of antisera to neutralize vaccine strains. Moreover, as combined vaccines have become the most common canine vaccines, many important viral pathogens could be neutralized along with vaccine strains during the testing process.

The detection of extraneous virus in vaccines by PCR would overcome these disadvantages of the culture method. In order to detect the homologous wild-type virus which contaminates vaccine preparations, primers were designed to distinguish wild-type (CPV type 2a and CPV type 2b) strains from vaccine
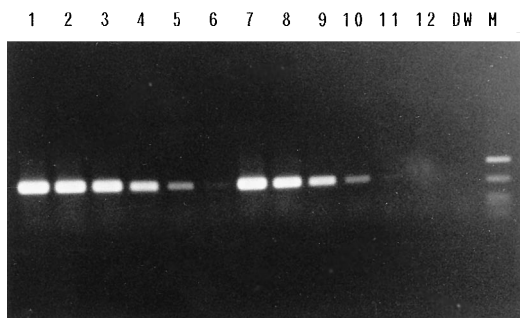

FIG. 3. Sensitivity for detection of CPV in PBS by PCR. Lanes 1 to 6, PCR products from CPV type 2a (the TDKeT9142 strain); lanes 7 to 12, PCR products from CPV type 2b (the Y-1 strain). Lanes 1 and 7, 1 $TCID_{50}$ per sample; lanes 2 and 8, 10^{-1} $TCID_{50}$ per sample; lanes 3 and 9, 10^{-2} $TCID_{50}$ per sample; lanes 4 and 10, 10^{-3} $TCID_{50}$ per sample; lanes 5 and 11, 10^{-4} $TCID_{50}$ per sample; lanes 6 and 12, 10^{-5} $TCID_{50}$ per sample. Lane DW, distilled water (negative control); lane M, molecular mass markers.
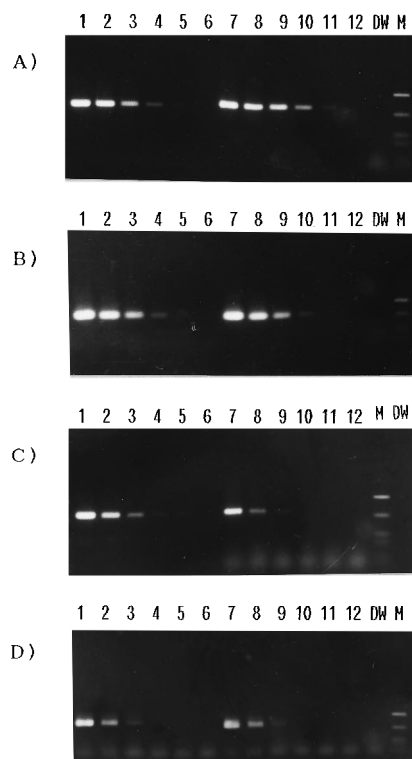

FIG. 4. Sensitivity for detection of wild-type CPV artificially mixed in vaccines. (A, B, C, and D) Results in vaccine A, B, C, and D, respectively. Lanes 1 to 6, PCR products from CPV type 2a (the TDKeT9142 strain); lanes 7 to 12, PCR products from CPV type 2b (the Y-1 strain). Lanes 1 and 7, 1 $TCID_{50}$ per sample; lanes 2 and 8, 10^{-1} $TCID_{50}$ per sample; lanes 3 and 9, 10^{-2} $TCID_{50}$ per sample; lanes 4 and 10, 10^{-3} $TCID_{50}$ per sample; lanes 5 and 11, 10^{-4} $TCID_{50}$ per sample; lanes 6 and 12, 10^{-5} $TCID_{50}$ per sample. Lane DW, distilled water (negative control); lane M, molecular mass markers.

strains. In the case of CPV, field strains went from the old type to the new type between 1979 and 1983. All 50 isolates collected in Japan and 48 of 51 isolates collected in the United States after 1983 belong to the new type (Table 2), while vaccine strains have remained the old type. Therefore, vaccine strains acquired natural markers as the old type, and nucleotide substitutions between old- and new-type DNAs have previously been defined (12). The 3'-terminal nucleotide of each primer, which is critical for polymerase initiation in PCR, is designed from the sequences of new-type viruses; therefore, these primers amplified DNA from new-type strains only. The results of PCR with all of the new-type strains from Japan showed that the sequences of these primers are highly conserved among new-type strains. A low rate of cross-amplifying was observed during a preliminary test which used only one differential primer in PCR with another of common sequence; that did not occur, however, when two differential primers were used.

Although no contamination by new-type CPV was detected by PCR in trial tests for extraneous virus in vaccines currently in use, the risk of contamination is always latent. Because a small dose of CPV does not show clear cytopathogenic effect in most cell cultures and parvoviruses are so resistant to disinfection, they could be carried and contaminate materials for vaccines in spite of great care.

Sample preparation for this PCR is minimal; vaccines to be tested are added directly to the PCR mixture, making this a very rapid and simple assay. Detection by PCR was extremely

sensitive as well as rapid after dilution in either PBS or vaccine. In the case of combined vaccines, sensitivities were somewhat lower than those for monovalent vaccines, perhaps because of some inhibitory substance for PCR in those vaccines. Their sensitivities, however, are much higher than that of the culture method, and they also allowed the detection of new-type virus in old-type vaccines. This model of a detection method for wild-type viruses in the presence of vaccine strains could be applied to many other viruses if the genetic markers of vaccine strains are defined and could also be used to detect defined heterologous viruses in vaccines if primers derived from the conserved sequences among heterologous viruses are used.

We propose a new concept of PCR-defined virus-specific pathogen-free vaccine, in addition to or instead of the present test for unspecified contaminating viruses by tissue culture, when all the important pathogens can be detected by PCR or other modern techniques.

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